Current Protocols in Food Analytical Chemistry

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FOREWORD

Accurate, precise, sensitive, and rapid analytical determinations are as essential in food science and technology as in chemistry, biochemistry, and other physical and biological sciences. In many cases, the same methodologies are used. How does one, especially a young scientist, select the best methods to use? A review of original publications in a given field indicates that some methods are cited repeatedly by many noted researchers and analysts, but with some modifications adapting them to the specific material analyzed. Official analytical methods have been adopted by some professional societies, such as the Official Methods of Analysis (Association of Official Analytical Chemists), Official Methods and Recommendation Practices (American Oil Chemists' Society), and Official Methods of Analysis (American Association of Cereal Chemists).

The objective of *Current Protocols in Food Analytical Chemistry* is to provide the type of detailed instructions and comments that an expert would pass on to a competent technician or graduate student who needs to learn and use an unfamiliar analytical procedure, but one that is routine in the lab of an expert or in the field.

What factors can be used to predetermine the quality and utility of a method? An analyst must consider the following questions: Do I need a proximate analytical method that will determine all the protein, or carbohydrate, or lipid, or nucleic acid in a biological material? Or do I need to determine one specific chemical compound among the thousands of compounds found in a food? Do I need to determine one or more physical properties of a food? How do I obtain a representative sample? What size sample should I collect? How do I store my samples until analysis? What is the precision (reproducibility) and accuracy of the method or what other compounds and conditions could interfere with the analysis? How do I determine whether the results are correct, as well as the precision and accuracy of a method? How do I know that my standard curves are correct? What blanks, controls and internal standards must be used? How do I convert instrumental values (such as absorbance) to molar concentrations? How many times should I repeat the analysis? And how do I report my results with appropriate standard deviation and to the correct number of significant digits? Is a rate of change method (i.e., velocity as in enzymatic assays) or a static method (independent of time) needed?

Current Protocols in Food Analytical Chemistry will provide answers to these questions. Analytical instrumentation has evolved very rapidly during the last 20 years as physicists, chemists, and engineers have invented highly sensitive spectrophotometers, polarometers, balances, etc. Chemical analyses can now be made using milligram, microgram, nanogram, or picogram amounts of materials within a few minutes, rather than previously when grams or kilograms of materials were required by multistep methods requiring hours or days of preparation and analysis. *Current Protocols in Food Analytical Chemistry* provides state-of-the-art methods to take advantage of the major advances in sensitivity, precision, and accuracy of current instrumentation.

How do chemical analyses of foods differ from analyses used in chemistry, biochemistry and biology? The same methods and techniques are often used; only the purpose of the analysis may differ. But foods are to be used by people. Therefore, methodology to determine safety (presence of dangerous microbes, pesticides, and toxicants), acceptability (flavor, odor, color, texture), and nutritional quality (essential vitamins, minerals, amino acids, and lipids) are essential analyses. *Current Protocols in Food Analytical Chemistry* is designed to meet all these requirements.

John Whitaker
Davis, California

Contributed by John Whitaker
Current Protocols in Food Analytical Chemistry (2001)

PREFACE

Accurate and state-of-the-art analysis of food composition is of interest and concern to a divergent clientele including research workers in academic, government, and industrial settings, regulatory scientists, analysts in private commercial laboratories, and quality control professionals in small and large companies. Some methods are empirical, some commodity specific, and many have been widely accepted as standard methods for years. Others are at the cutting edge of new analytical methodology and are rapidly changing. A common denominator within this diverse group of methods is the desire for detailed descriptions of how to carry out analytical procedures. A frustration of many authors and readers of peer-reviewed journals is the brevity of most Materials and Methods sections. There is editorial pressure to minimize description of experimental details and eliminate advisory comments. When one needs to undertake an analytical procedure with which one is unfamiliar, it is prudent to communicate first-hand with one experienced with the methodology. This may require a personal visit to another laboratory and/or electronic or phone communication with someone who has expertise in the procedure. An objective of *Current Protocols in Food Analytical Chemistry* is to provide exactly this kind of detailed information which personal contact would provide. Authors are instructed to present the kind of details and advisory comments they would give to a graduate student or technician who has competent laboratory skills and who has come to them to learn how to carry out an analytical procedure for which the author has expertise.

Some basic food analytical methods such as determination of Brix, pH, titratable acidity, total proteins, and total lipids are basic to food analysis and grounded in procedures which have had wide-spread acceptance for a long time. Such methods cannot be ignored and are included in this manual. Others, such as analysis of cell-wall polysaccharides (Chapter E3), analysis of aroma volatiles (Chapter G1), and compressive measurement of solids and semisolids (Chapter H2), require use of advanced chemical and physical methods and sophisticated instrumentation. These methods are particularly prone to rapid change and evolution. *Current Protocols* capitalizes on today's electronic communication technologies and provides a mechanism for updates, additions, and revisions. The publication is available in loose-leaf binder, CD-ROM, or Online formats. Supplements are published quarterly. Users have the opportunity to provide feedback so that individual units can be clarified, modified, and expanded. Thus *Current Protocols in Food Analytical Chemistry* should be viewed as a dynamic resource which will be constantly up-dated.

In organizing *Currrent Protocols in Food Analytical Chemistry* we chose to categorize on a disciplinary rather than a commodity basis. Included are chapters on water, proteins, enzymes, lipids, carbohydrates, colors, flavors, and textural components. We have made an effort to select methods which are applicable to all commodities. However, it is impossible to address the unique and special criteria required for analysis of all commodities and all processed forms. There are several professional and trade organizations which focus on their specific commodities, e.g., cereals, wines, lipids, fisheries, and meats. Their methods manuals and professional journals should be consulted, particularly for specialized, commodity-specific analyses.

With respect to our Editorial Board, we have selected scientists who are widely regarded as being authorities in their field. Their research productivity is impressive, and they are all professors who are experienced in training undergraduate and graduate students, technicians, post-doctoral students, and visiting scientists in analytical procedures. This common experience provides insight regarding the types of experimental details needed to clarify how procedures are to be conducted.

Contributed by Ronald E. Wrolstad, Terry E. Acree, Haejung An, Eric A. Decker, Michael H. Penner, David S. Reid, Steven J. Schwartz, Charles F. Shoemaker, Denise M. Smith, and Peter Sporns
Current Protocols in Food Analytical Chemistry (2003) iii-vi

HOW TO USE THIS MANUAL

Format and Organization

This publication is available in looseleaf, CD-ROM, and Online formats. For loose-leaf purchasers, a binder is provided to accommodate the growth of the manual via the quarterly update service. The looseleaf format of the binder allows easy insertion of new pages, units, and chapters that are added. The index and table of contents are updated with each supplement. Purchasers of the CD-ROM and Intranet versions receive a completely new disc every quarter and should dispose of their outdated discs. The material covered in all versions is identical.

Subjects in this manual are organized by sections and chapters, and protocols are contained in units. Units generally describe a method and include one or more protocols with listings of materials, steps and annotations, recipes for unique reagents and solutions, and commentaries on the "hows" and "whys" of the method; there are also "overview" units containing theoretical discussions that lay the foundation for subsequent protocols. Page numbering in the looseleaf version reflects the modular arrangement by unit; for example, page D2.1.1 refers to Section D (Lipids), Chapter D2 (Lipid Oxidation/Stability), *UNIT D2.1* (Measurement of Primary Lipid Oxidation Products), page 1 of that particular unit.

Many reagents and procedures are employed repeatedly throughout the manual. Instead of duplicating this information, cross-references among units are used extensively. Cross-referencing helps to ensure that lengthy and complex protocols are not overburdened with steps describing auxiliary procedures needed to prepare raw materials and analyze results.

Certain units that describe commonly used techniques and recipes are cross-referenced in other units that describe their application.

Introductory and Explanatory Information

Because this publication is first and foremost a compilation of laboratory techniques in food analytical chemistry, we have not offered extensive instructive material. We have, however, included explanatory information where required to help readers gain an intuitive grasp of the procedures. Some chapters begin with overview units that describe the state of the art of the topic matter and provide a context for the procedures that follow. Section and unit introductions describe how the protocols that follow connect to one another, and annotations to the actual protocol steps describe what is happening as a procedure is carried out. Finally, the Commentary that closes each protocol unit describes background information regarding the historical and theoretical development of the method, as well as alternative approaches, critical parameters, troubleshooting guidelines, anticipated results, and time considerations. All units contain cited references and many indicate key references to inform users of particularly useful background reading, original descriptions, or applications of a technique.

Protocols

Many units in the manual contain groups of protocols, each presented with a series of steps. The *Basic* Protocol is presented first in each unit and is generally the recommended or most universally applicable approach. *Alternate* Protocols are given where different equipment or reagents can be employed to achieve similar ends, where the starting material requires a variation in approach, or where requirements for the end product differ from those in the Basic Protocol. *Support* Protocols describe additional steps that are

required to perform the Basic or Alternate Protocols; these steps are separated from the core protocol because they might be applicable to other uses in the manual, or because they are performed in a time frame separate from the Basic Protocol steps.

Reagents and Solutions

Reagents required for a protocol are itemized in the materials list before the procedure begins. Many are common stock solutions, others are commonly used buffers or media, whereas others are solutions unique to a particular protocol. Recipes for the latter solutions are supplied in each unit, following the protocols (and before the commentary) under the heading Reagents and Solutions. It is important to note that the *names* of some of these special solutions might be similar from unit to unit (e.g., SDS sample buffer) while the *recipes* differ; thus, make certain that reagents are prepared from the proper recipes. On the other hand, recipes for commonly used stock solutions and buffers are listed once in *APPENDIX 2A*. These universal recipes are cross-referenced parenthetically in the materials lists rather than repeated with every usage.

Commercial Suppliers

In some instances throughout the manual, we have recommended commercial suppliers of chemicals, biological materials, or equipment. This has been avoided wherever possible, because preference for a specific brand is subjective and is generally not based on extensive comparison testing. Our guidelines for recommending a supplier are that (1) the particular brand has actually been found to be of superior quality, or (2) the item is difficult to find in the marketplace. The purity of chemical reagents frequently varies with supplier. Generally *reagent grade* chemicals are preferred. Special care must be paid to procedures that require dry solvents. Different suppliers provide special anhydrous grade solvents which may vary in water content depending on the supplier. Addresses, phone numbers, facsimile numbers, and web addresses of all suppliers mentioned in this manual are provided in the *SUPPLIERS APPENDIX*.

Safety Considerations

Anyone carrying out these protocols will encounter hazardous or potentially hazardous materials including toxic chemicals and carcinogenic or teratogenic reagents. Most governments regulate the use of these materials; it is essential that they be used in strict accordance with local and national regulations. Cautionary notes are included in many instances throughout the manual, but we emphasize that users must proceed with the prudence and precaution associated with good laboratory practice, and that all materials be used in strict accordance with local and national regulations.

Reader Response

Most of the protocols included in this manual are used routinely in our own laboratories. These protocols work for us; to make them work for you we have annotated critical steps and included critical parameters and troubleshooting guides in the commentaries to most units. However, the successful evolution of this manual depends upon readers' observations and suggestions. Consequently, a self-mailing reader-response survey can be found at the back of the manual (and is included with each supplement); we encourage readers to send in their comments.

ACKNOWLEDGMENTS

This manual is the product of dedicated efforts by many of our scientific colleagues who are acknowledged in each unit and by the hard work of the Current Protocols editorial staff at John Wiley and Sons. The publisher's commitment and continuing support for a food analytical chemistry manual were essential for realizing this ambitious project. We are extremely grateful for the critical contributions by Ann Boyle and Elizabeth Harkins (Series Editors) who kept the editors and the contributors on track and played a key role in bringing the entire project to completion. Other skilled members of the Current Protocols staff who contributed to the project include Tom Cannon Jr., Davide Dickson, Michael Gates, Tuan Hoang, Alice Ro, Liana Scalettar, Mary Keith Trawick, and Joseph White. The extensive copyediting required to produce an accurate protocols manual was ably handled by Allen Ranz, Amy Fluet, Tom Downey, and Susan Lieberman.

KEY REFERENCES

Association of Official Analytical Chemists (AOAC): Official Methods of Analysis, 2000. AOAC, Arlington, Va.

A compilation of analytical methods which have been collaboratively tested and approved as official methods by the AOAC. Available as a monograph, in loose-leaf binder or CD-ROM.

Food Chemicals Codex. Fourth Edition, 1996. Committee on Food Chemicals Codex, National Academy of Sciences, National Research Council, Washington D.C. National Academy Press. Available on CD-ROM, CRC Press, Boca Raton, Fla.

Provides quality standards for an extensive list of food chemicals along with chemical and physical methods for their determination.

Journal of the Association of Official Analytical Chemists. AOAC. Arlington, Va.

Peer-reviewed journal providing original articles on methods of analysis, compositional data and collaborative studies.

AACC Approved Methods, 10th Edition, 2000. American Association of Cereal Chemists, St. Paul, Minn.

Standardized, approved methods for analysis of cereal grains and cereal-based ingredients. Available in printed form or CD-ROM.

American Journal of Enology and Viticulture. American Society of Enology and Viticulture, Davis, Calif.

Journal contains peer-reviewed articles giving analytical methods for analysis of wines and grape products.

American Oil Chemists Association (AOCS): Official and Tentative Methods of Analysis. American Oil Chemists Society, Champaign, Ill.

A compilation of standard methods for the analysis of fats and oils approved by the American Oil Chemists Association.

American Society of Brewing Chemists. Methods of Analysis of the American Society of Brewing Chemists 1992. 8th edition, ASBC, St. Paul, Minn.

Standard methods of analysis recommended by the American Society of Brewing Chemists.

Ronald E. Wrolstad, Terry E. Acree, Haejung An,
Eric A. Decker, Michael H. Penner, David S. Reid,
Steven J. Schwartz, Charles F. Shoemaker, Denise M. Smith,
and Peter Sporns

Gravimetric Determination of Water by Drying and Weighing

Water (moisture) in a sample is measured gravimetrically by determining the weight loss in a sample after it has been placed in an appropriate oven (convection, vacuum, or microwave) for a given time. In addition, there are automatic moisture analyzers available that utilize infrared lamps as a heat source. These types of moisture analyzers are fast but many times are matrix dependent, which requires some trial-and-error testing to determine the correct settings (power and time). Water and moisture are used interchangeably in the description of these protocols. In addition, it is assumed in the gravimetric method that only water is removed in the drying process, when in fact there may be volatile loss in some samples.

Although the measurement of weight loss due to evaporation of water is frequently used to calculate moisture content, it should be pointed out that the value obtained may not be a true measure of water content. In some samples, only a proportion of the water present is lost at the drying temperature. The balance (bound water) is difficult to remove completely. In addition, the water lost may actually increase as the temperature is raised. Some samples with high fat content may exhibit volatile oil loss at drying temperatures of 100°C. Weight loss may also be dependent on such factors as particle size, weight of samples used, type of dish used, and temperature variations in the oven from shelf to shelf. Thus, it is important to compare results obtained using the same drying conditions.

This unit provides three protocols for which there are established procedures for various matrices. The Basic Protocol describes water removal and quantitation after a sample is placed in a convection oven. It is probably the method of choice when one does not know which method to choose when dealing with an unknown matrix, or when one looks at samples that foam excessively in the vacuum oven method or "react," such as popcorn under vacuum. Alternate Protocol 1 describes water removal and quantitation after a sample is placed in a vacuum oven. Because it is at reduced pressure, drying times are slightly reduced compared to the convection method. In addition, drying temperatures <100°C are possible, which is important for samples that may decompose at higher drying temperatures. Alternate Protocol 2 describes water removal using a microwave source where such analyzers measure and calculate loss automatically.

MEASURING MOISTURE USING A CONVECTION OVEN

BASIC PROTOCOL

Water is measured in a sample by determining the loss in weight for the sample after it has been dried in a convection oven. The method requires only a small amount of homogeneous sample and can measure an effective range of 0.01% to 99.99% water.

Materials

Homogeneous sample
Convection oven capable of maintaining a temperature of 103° ± 2°C
Aluminum weighing dishes (with or without covers)
Desiccator with desiccant
Balance capable of measuring ±0.1 mg

1. Set the temperature of a convection oven to 105°C.
2. Dry an aluminum weighing dish (and cover, if used) ≥1 hr at 105°C. Cool and store dried dish in a desiccator. Cool ≥30 min before using.

Covered weighing dishes are useful when analyzing samples that splatter. Weighing dishes without covers may otherwise be preferred, as they are disposable.

Contributed by Rennie P. Ruiz
Current Protocols in Food Analytical Chemistry (2001) A1.1.1-A1.1.6

3. Weigh empty, dried dish (with cover, if used) to nearest 0.1 mg.
4. Add 3 to 10 g homogeneous sample to dish and weigh (with cover, if used) to nearest 0.1 mg.
5. Place dish with sample into oven and dry 4 hr.
6. Remove dish with dried sample and cool 30 min in a desiccator.
7. Weigh cooled dish with dried sample.
8. Return dish with sample to oven, dry for another hour, cool, and reweigh.
9. If weight has not changed, test is done. If weight is lower, continue drying for 1-hr periods and reweighing until constant weight is achieved.

   Depending on the sample, drying times can be ≥16 hr.
10. Calculate moisture as the percent loss in weight after drying.

ALTERNATE PROTOCOL 1

MEASURING MOISTURE USING A VACUUM OVEN

Water is measured in a sample by determining the loss in weight for the sample after it has been dried in a vacuum oven. The method requires only a small amount of homogeneous sample and can measure an effective range of 0.01% to 99.99% water. As indicated earlier, the vacuum oven method allows one to dry samples at temperatures <100°C. Some matrices may require drying at 70°C (Table A1.1.1), but the following procedure addresses a drying temperature between 95° and 100°C.

Additional Materials *(also see Basic Protocol)*

Sulfuric acid
Vacuum oven capable of withstanding vacuum ≤100 mmHg and maintaining temperature of 95° to 100°C
Vacuum pump capable of maintaining a vacuum ≤100 mmHg
500-ml gas washing bottle

CAUTION: Sulfuric acid is hazardous, causing severe burns, and must be handled and disposed of accordingly. See Safety Appendix (*APPENDIX 2B*).

1. Set up a vacuum oven by connecting the oven air outlet to a vacuum pump. Connect the oven air inlet to a 500-ml gas washing bottle that contains sulfuric acid. Set oven temperature to 95° to 100°C.

   The sulfuric acid is used to dry the air that is admitted during drying.
2. Dry an aluminum weighing dish (and cover, if used) ≥1 hr at 100°C. Cool and store dried dish in a desiccator. Cool ≥30 min before using.

   Covered weighing dishes are useful when analyzing samples that splatter. Weighing dishes without covers may otherwise be preferred, as they are disposable.
3. Weigh empty, dried dish (with cover, if used) to nearest 0.1 mg.
4. Add 3 to 10 g homogeneous sample to dish and weigh (with cover, if used) to nearest 0.1 mg.
5. Place dish with samples into vacuum oven and close oven door.
6. Close both air inlet and outlet valves to oven. Start vacuum pump and slowly evacuate air from inside the oven by opening the oven outlet valve. Once pressure is ≤100 mmHg, open oven air inlet slightly so that air is drawn into the gas washing bottle,

Table A1.1.1 Recommended Moisture Protocols

Matrix	Recommended oven type	Conditions
Animal feeds	Vacuum	2 hr at 95°-100°C
Ice cream/frozen desserts	Convection	3.5 hr at 100°C
Cheese	Vacuum	4 hr at 100°C
Dried milk	Vacuum	5 hr at 100°C
Seafood	Convection	4 hr at 100°C
Meat	Convection	16-18 hr at 100°C
Meat and poultry products	Microwave	Mode 2
Dried eggs	Convection	5 hr at 98°-100°C
Frozen french-fried potatoes	Convection	16 hr at 103°C
Tomato/tomato products	Microwave	Mode 1, 4 min
Canned vegetables	Vacuum	Constant weight at 70°C
Dried fruits	Vacuum	6 hr at 70°C
Fruit/fruit products	Vacuum	Constant weight at 70°C
Nuts/nut products	Vacuum	5 hr at 95°-100°C
Flour	Vacuum	5 hr at 98°-100°C
Sugars	Convection	3 hr at 70°C
Sugars	Vacuum	3 hr at 100°C

through the sulfuric acid, and into the oven at a rate of ~2 bubbles/sec. Dry sample 4 hr.

7. Isolate vacuum pump by closing oven outlet valve and carefully admitting dried air into oven (by slowly opening air inlet valve fully), increasing the pressure inside oven until the door can be easily opened. Remove drying dish with sample and cool 30 min in a desiccator.
8. Weigh cooled dish with sample.
9. Repeat steps 5 to 8, except dry for 1 hr.
10. If weight has not changed, test is done. If weight is lower, continue drying for 1-hr periods and reweighing until constant weight is achieved.

    Most samples take ~4 hr to dry.

11. Calculate moisture as the percent loss in weight after drying.

MEASURING MOISTURE USING A MICROWAVE MOISTURE ANALYZER

ALTERNATE PROTOCOL 2

Water is measured in a sample using a microwave moisture analyzer that uses microwave energy to remove water. The instrument weighs the sample both before and after drying and calculates the percent moisture automatically. The analyzer can measure an effective range of 0.1% to 99.9% water, but may have problems at the lower range due to burning or scorching, especially if there is a high sugar content.

The microwave moisture analyzer allows one to dry a sample by two modes. Mode 1 dries a sample at a specific power setting for a given time period. Mode 2 dries a sample at a specific power setting using a time period that is determined by the analyzer itself, ending the run when no weight change is achieved within a preset time interval criterion. This protocol looks only at Mode 1 operation.

Materials

Homogeneous sample
Microwave moisture analyzer (e.g., AVC-80, CEM Corp.)
Fiberglass sample pads

1. Turn on a microwave analyzer and let warm up 30 min.
2. Set analyzer for Mode 1 operation, setting power to 100%, display to % moisture, and drying time interval to 4 min.
3. Dry two fiberglass sample pads by placing them on the balance ring, dropping cover over pads, and closing analyzer door.
4. Press "tare," wait for reading to stabilize, then press "run" to start drying program.
5. When drying is done, press "tare," wait for reading to stabilize, and then remove fiberglass pads.
6. Deposit ~1 to 2 g sample onto the rough side of one fiberglass pad. If sample is a liquid, use a pipet to cover as much of the pad as possible. If a semisolid, use a spatula to spread as evenly as possible. Avoid lumps. Apply sample as quickly as possible to avoid loss of moisture during application.
7. Place second pad, rough side down, on top of sample and place pads and sample on balance ring in analyzer.
8. Drop cover over sample, close analyzer door, wait for displayed weight to stabilize, and then start the drying process by pressing "run."

   Program ends after 4 min and calculates percent moisture automatically.

9. Remove pads and inspect dried sample for areas of scorching, burning, or wetness. If these conditions exist, repeat test, being careful to spread the sample as evenly as possible onto pad.

   Scorching and burning may indicate that the power setting is too high, and wetness may indicate improper spreading of the sample.

   When running many samples, several sets of pads can be placed inside the analyzer so that they may dry while samples are actually being run. This way, separate sets of pads do not have to be dried individually, and it is possible to begin at step 5 to tare the dried pads before use.

COMMENTARY

Background Information

Moisture analyses are important because samples contain water either as chemically combined hydrates or as occluded surface-adsorbed moisture. Water is an inherent part of most biological substances and constitutes >90% of the fresh weight of some plant materials. To afford reproducible analytical results, samples are usually dried before analysis and the percentage composition of the sample is then calculated on a dry basis.

Gravimetric analyses for moisture are probably the easiest procedures to use, as there are no chemicals to prepare. Manpower is at a minimum, requiring only a weighing step before and after heating in an appropriate oven. As an example, ~29 of the 35 moisture methods recognized by the Association of Official Analytical Chemists (AOAC) for nutritional labeling are gravimetric methods using some sort of a drying oven (Sullivan and Carpenter, 1993). The only concern with gravimetric methods, using either a vacuum or convection oven, is the amount of time required for drying. Most samples take ~4 hr in a vacuum oven. Use of a convection oven, at 1 atm and slightly higher temperature, could add as much as 16 hr depending on the sample (e.g., unpopped corn kernels). Because of the long drying times,

analyzing as many samples as possible at one time can maximize sample throughout.

Microwave moisture analyzers are the next step in gravimetric analyses by focusing on much shorter drying times. Currently there are only a few matrices recognized by the AOAC for use in the microwave drying method (Sullivan and Carpenter, 1993). Although there hasn't been a collaborative study for AOAC approval to support other matrices, the microwave moisture analyzer has been used successfully in such matrices as grains (Okabe et al., 1973) and flours (Davis and Lai, 1984).

The choice of which protocol to use will depend on what type of matrix is being tested. As a first suggestion, Table A1.1.1 is provided based on the matrices and methodologies currently recognized by the AOAC (Sullivan and Carpenter, 1993). As one can see, many matrices can be run by more than one gravimetric technique. The method used will depend on the matrix or on the end use of the dried sample. For a general review of all methods, Karmas (1980) has written an evaluation with emphasis on food matrices.

Critical Parameters and Troubleshooting

All gravimetric methods require only a very small amount of sample (between 1 and 10 g depending on the moisture level). Therefore, sample homogeneity is very critical and special care must be given to sample blending before subsampling for analysis. The method used for homogenization will depend on the type of sample being analyzed. Blenders, homogenizers, mincers, food processors, and graters are currently available for dry, moist, and very wet samples. Because these devices produce heat, it is very important not to overhomogenize, which causes moisture to be lost. Once a sample has been properly homogenized, it is important to either run the sample for moisture as soon as possible or transfer it to either a dry glass or plastic container with a well-fitting closure or seal.

In the gravimetric method, low-moisture samples will only produce a very small loss of weight upon drying. It is important not to contaminate the samples when drying them or transferring them to the oven or desiccator. Do not handle dishes with bare hands; use tongs or Kimwipes. Assuming that good weighing techniques are used, the other source of problems stems from the drying portion of the test. Samples that foam excessively or splatter when heated require the use of covered weighing dishes. Covers also prevent cross-contamination to neighboring samples in the oven should splattering occur. Very high-moisture samples can also be dried, after initial weighing, in a convection oven to reduce the volume before drying in a vacuum oven. This may help with samples that foam and spill over the sides of the weighing dish. For the microwave oven method, how the sample is placed on the fiberglass pad is critical. Samples must be spread out as smoothly as possible with the spatula. Lumps and thick areas of sample on the pad may not be completely dried at the end of the test. Improper spreading of the sample, or two high of a power setting, may also lead to areas of scorching or burning on the pad.

Anticipated Results

The concentration range for gravimetric analyses of moisture is between 0.01% and 99.99%. Relative standard deviations of ±1% are achievable.

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Karl Fischer Titration

BASIC PROTOCOL

Unlike gravimetric measurements (*UNIT A1.1*), which are indirect methods that assume that all volatiles removed are water, the Karl Fischer titration is a direct method that is almost specific for water. The method is especially useful for low moisture levels (<1%), and levels <0.01% are achievable. The Karl Fischer titration method is especially useful for samples that may be high in sugar, or high in both reducing sugar and protein, which may decompose in the gravimetric methods. The method is a titration of water with an anhydrous methanol solution containing iodine, sulfur dioxide and excess pyridine. The titration is based on the reaction between iodine and sulfur dioxide that will occur only if water is present:

$$I_2\text{-Pyr} + SO_2\text{-Pyr} + \text{Pyr} + H_2O \rightarrow SO_3\text{-Pyr} + 2\text{Pyr}H^+I^-$$

where Pyr represents pyridine. The product, SO_3-Pyr reacts further with the methanol to form the methylsulfate anion:

$$SO_3\text{-Pyr} + CH_3OH \rightarrow \text{Pyr}H^+CH_3SO_4^-$$

From these equations it can be seen that each mole of water requires one mole of I_2. In a visual endpoint Karl Fischer titration, a sample is titrated with the Karl Fischer reagent until a permanent iodine color (indicating that all water has been reacted) is observed. Because of other reaction products, the color change is usually from a yellow to a brownish color, which may be difficult to detect visually. Highly colored samples may affect the visual end point as well. A much sharper end point, known as the "dead stop" end point, can be obtained if the titration is done electrometrically. Here, two small platinum electrodes dip into the titration cell, a small constant voltage is impressed across these electrodes, and any current that flows is measured with a galvanometer. At the end point of the titration the current either goes to a minimum or else increases suddenly from nearly zero. Commercially available Karl Fischer instruments incorporate semiautomatic microprocessors based on this principle.

Only the volumetric Karl Fischer titration (see Basic Protocol) will be discussed in this unit. In addition, it is assumed that the sample being tested is a low-moisture solid, where it is necessary to weigh out the sample, add it to a sealable flask of anhydrous methanol to "extract" the water, and measure the amount of extracted water.

Materials

Karl Fischer reagent (e.g., Sigma)
Water standard
Karl Fischer solvent: anhydrous methanol (dried using molecular sieves)
Sample for analysis, homogeneous

Karl Fischer titration assembly, automatic (Metrohm 701 or 758, Brinkmann or equivalent)
1-ml syringes (or 10-ml for low-moisture samples)
Dried, clean 10- and 50-ml volumetric flasks
Balance capable of measuring ±0.1 mg

Standardize Karl Fischer reagent

1. Set up titration assembly per manufacturer's instructions.

   Most titrators have a sealed titration vessel where liquid samples are injected and the titrator adds the Karl Fischer reagent until a stable endpoint is achieved. This protocol

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assumes such a case where the endpoint is indicated as that volume of Karl Fischer reagent required to react with all water present. No matter which volumetric titration instrument is used, it is necessary to standardize the Karl Fischer reagent and run blanks on the Karl Fischer solvent (methanol).

Commercially available Karl Fischer reagent is stabilized and can be obtained free of pyridine, which is toxic. It can also be prepared in the laboratory; however, this requires the use of SO_2, a poisonous, liquified gas.

2. Add dried Karl Fischer solvent to the titration assembly vessel so that electrodes are submerged, and seal vessel.

   Remember to reseal the container containing the Karl Fischer solvent immediately after removing the amount needed. The vessel will allow many titrations before it has to be emptied, cleaned, dried and reassembled.

3. Run titrator, without adding any sample, to neutralize any water from the Karl Fischer solvent or moisture in vessel.

4. Accurately weigh 125 mg of water and dilute to volume in a dried, clean 10-ml volumetric flask with dried Karl Fischer solvent.

5. Draw up and discard an aliquot of water standard in a syringe to remove any moisture in the syringe. Repeat.

   Remember to reseal flask immediately upon removing any sample.

6. Inject 1-ml aliquot of the water standard into the vessel and titrate to determine conversion factor.

   This standard must be run daily or whenever a new batch of Karl Fischer reagent or solvent is being used.

7. Draw up and discard an aliquot of the Karl Fischer solvent to remove any moisture in the syringe. Repeat.

8. Inject a 1-ml aliquot of dried Karl Fischer solvent into the vessel of the titration assembly and titrate to determine blank correction.

   Duplicates are recommended.

9. Using measured blank result, correct readings for Karl Fischer solvent and determine standardization concentration for the Karl Fischer reagent (mg H_2O/ml Karl Fischer reagent).

   Most commercial Karl Fischer reagents run ~5 mg H_2O/ml reagent.

Extract and measure sample moisture

10. Weigh, to the nearest 0.1 mg, a quantity of the homogeneous sample containing ~100 mg water into a dried, 50-ml volumetric flask. Dilute to volume with dried Karl Fischer solvent.

11. Add a small stir bar, seal flask, and stir for 10 min. Let sample settle.

12. After solids have settled out, draw up and discard an aliquot of the solvent containing the extracted water, to remove any extraneous moisture from the syringe. Repeat.

13. Inject a 1-ml aliquot of the solvent containing the extracted water into the vessel and start titration.

    Use larger syringe to inject larger volumes for low moisture samples. Duplicates are recommended.

14. Calculate percent moisture using the equation:

$$\% \text{ Moisture} = [(M - B)(C/1000/W) \times 50] \times 100$$

where M is ml of Karl Fischer titrant from the 1-ml injection in step 13, B is ml of Karl Fischer titrant for the blank in step 8, and C is the standardization amount (mg H_2O/ml Karl Fischer titrant) in step 6, W is the weight of sample in grams in step 10, 1000 converts mg to g, 50 is a dilution factor (50 ml total volume, 1 ml aliquot used), and 100 converts to percentage.

COMMENTARY

Background Information

The Karl Fischer titration, nearly specific for water, is very important when looking at very low levels of moisture. It is especially useful for the determination of water in organic compounds and for some samples that are partially decomposed at temperatures used in the gravimetric methods (*UNIT A1.1*). Unlike gravimetric methods, the Karl Fischer titration does not provide a dried sample that may be used for further tests. Because it is a sensitive method, contact with any moisture, even from the surrounding environment, must be eliminated as much as possible. Commercial instruments are available with all-glass joints and seals, which keep moisture contamination to a minimum. In addition, these instruments can be fitted with openings into the reaction vessel where samples can be added directly. The disadvantage of "direct" addition of samples is the presence of particulates and decomposition materials in the reaction vessel, which eventually will have to be removed more frequently.

Commercial instruments can be classified into either coulometric or volumetric Karl Fischer titrators. In coulometric Karl Fischer measurements, one measures the amount of water that will undergo a reaction at an electrode with 100% efficiency by measuring the amount of electricity (in coulombs) necessary to effect complete reaction (in this case, between water and the Karl Fischer reagent). The advantage is that standard solutions are unnecessary here. Sensitivity is very high (~10 µg water) and is very useful for trace level determinations. In the volumetric Karl Fischer titration, the analysis is based on the measurement of the volume of a standard solution (Karl Fischer reagent) that must be added to react with the water present. From the volume of the reagent of known concentration that has been added to reach the end point, the concentration of water may be determined. Volumetric Karl Fischer titrators require that the Karl Fischer reagent be standardized, but moisture levels from 10 ppm up to 100% can be measured. Which type of titrator to choose depends on individual needs (trace or high-level measurements) and budget as coulometric Karl Fischer titrators generally cost more than volumetric Karl Fischer titrators. Though not as sensitive at very low amounts of moisture compared to coulometric Karl Fischer titrators, volumetric Karl Fischer titrations allow one a larger dynamic range to measure.

Though the protocol was written for a dry solid, it should also be mentioned that liquid samples can also be run (Firestone, 1998). Liquid samples can be added directly to the vessel itself or can be treated as a solid with an extraction step in the Karl Fischer solvent as presented in the protocol. The preference for moisture analyses is the gravimetric method (*UNIT A1.1*) largely due the higher costs of equipment and the use of chemicals in the Karl Fischer method. Currently one observes very few applications of the Karl Fischer method on food products published by the AOAC for nutritional labeling purposes. In particular, these food products are cocoa, cocoa products, confectionery coatings, and molasses (Sullivan and Carpenter, 1993).

Critical Parameters and Troubleshooting

Like the gravimetric methods, the Karl Fischer titration method requires only a very small amount of sample (between 1 and 10 g depending on the moisture level). Therefore, sample homogeneity is very critical and special care must be given to sample blending before subsampling for analysis. The method used for homogenization will depend on the type of sample being analyzed. Blenders, homogenizers, mincers, food processors, and graters are currently available for dry, moist, and very wet samples. Because these devices produce heat, it is very important not to overhomogenize, thereby causing moisture to be lost. Once a sample has been properly homogenized, it is

important to either run the sample for moisture as soon as possible or transfer to either a dry glass or plastic container with a well fitting closure or seal.

Dried glassware (volumetric flasks, reaction vessel, syringes) and reagents are essential in this test. Seals on the titration vessel must be checked as moisture from the atmosphere may affect titration results. Drying tubes should always be used and checked frequently. All bottles and flasks should be resealed immediately after opening whenever sample aliquots are taken. Samples should be weighed quickly in the flask, Karl Fischer solvent added immediately, and the flask sealed quickly to prevent any possible moisture pickup. Care must be taken in choosing the appropriate size of sample to test. Too large a weight of a high-moisture sample will exceed the volume limits of the titrator used. Too small a weight of a low-moisture sample may not be measurable with the titrator used.

The Karl Fischer reagent is not very stable and must be standardized frequently. The commercially available Karl Fischer reagent can be purchased as a single solution and is more stable.

Interferences may occur with samples that contain iodine-reducing components. Samples that contain high levels of ketones or aldehydes, especially where low moisture levels are to be measured, may present a problem as these compounds may generate water.

Anticipated Results

Levels of moisture <0.01% are measurable and one can expect a relative standard deviation of ±1%.

Time Considerations

The Karl Fischer titration requires ~20 to 45 min (depending on sample being tested) to complete calibration, blank determination, sample equilibration (extraction in Karl Fischer solvent), and final testing.

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Application of Low-Resolution NMR for Simultaneous Moisture and Oil Determination in Food (Oilseeds)

There are many instances in the agriculture and food industry when commercial transactions, process and quality control decisions, or plant-breeding selection rely on the determination of moisture and oil levels. Accurate and precise simultaneous measurement of moisture and oil can be performed reliably, routinely, and rapidly (e.g., <30 sec) on mature seeds, grains, and nuts using an affordable benchtop pulsed NMR analyzer. The method is based on the detection of hydrogen that is present in the moisture and oil contained in the sample and on the different properties of hydrogen depending on its physical state or environment. Analysis by NMR is totally nondestructive, and therefore can be repeated any number of times on the same sample. Since both moisture and oil are determined for the same sample at the same time, errors due to separate sample handling and result recording are eliminated. In the case of oilseeds, the commercial value of the product depends mainly on the oil content as long as moisture levels are not so high as to cause spoilage. If the total moisture is measured at the same time as oil determination, the oil can be reported on an "as is" weight basis, or corrected by computer to dry weight or any another standard moisture level common to the particular industry or country.

This NMR protocol for simultaneous moisture and oil determination in oilseeds, grains, and nuts has been standardized internationally in International Standard Organization (ISO) 10565 (1999). This applies to mature seeds with preservation-level moisture content (e.g., <10% for canola and sunflower seed, <14% for soybean).

STRATEGIC PLANNING

A prerequisite for this method is mature samples (e.g., seeds, grains, nuts) that are not physically wet. It is known that oilseeds with the normal hydration content at levels suitable for seed storage are appropriate. All moisture must be in association with starches and other solids in the matrix in order for the standard NMR measurement to be applicable. In this state, the NMR T_2 relaxation time is on the order of tens of microseconds, whereas the oil is on the order of hundreds of milliseconds. If present, free moisture contributes to the oil signal when measured by the standard spin echo pulse sequence and therefore causes an error in measured moisture and oil values. The upper range for moisture content in certain oilseeds that still allow accurate NMR results has been published (Rubel, 1994). For example, canola and sunflower seed must have <10% and soybean <14% total moisture; otherwise, samples must be dried of surface water, excess absorbed moisture, or cytoplasmic moisture, as would be present in immature seeds, cereals, and nuts. Free moisture can be removed and quantified in the process, by measuring weight loss upon drying for a short period (i.e., 1 hr at 103°C in a drying oven).

Calibration for Percent Moisture and Oil

Calibration for percent moisture and oil in oilseeds is best done with real samples (i.e., seed, nuts, and grains) that have known moisture and oil contents as determined by appropriate reference methods (see below). The set of standards must have moisture and oil contents that at least span the range of all moisture contents likely to be encountered in unknown samples. Extrapolation of the calibration line, in order to measure samples that apparently fall outside the range of calibration values, is not recommended. Reference samples for moisture do not need to have known oil contents. Conversely, reference

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samples for oil do not need to have known moisture contents, as long as the moisture content does not exceed the limits discussed above (see Commentary for an explanation of the NMR measurement). Reference values for moisture content are commonly determined by Karl Fischer titration (*UNIT A1.2*) or weight-loss on drying by various methods (e.g. *UNIT A1.1*), and each has inherent advantages and disadvantages. The accuracy of the reference method ultimately limits the accuracy of the secondary NMR calibration line. Precision of the NMR method is the same regardless of calibration method and is limited by the stability and signal-to-noise of the NMR instrument.

Calibration for percentage oil can actually take two forms: primary calibration by use of weighed quantities of oil isolated from the oilseed of interest, or secondary calibration using oilseeds of known oil content, as determined by a reference method and typically solvent (hexane) extraction. One of these strategies must be selected prior to beginning a program of measuring unknown samples. This unit includes a series of alternate steps for either primary (see Basic Protocol, steps 10a to 14a) or secondary (steps 10b to 14b) calibration. Each has its advantages and disadvantages.

Primary calibration with increasing weights of oil is the most accessible, because new calibration standards can easily be reproduced as long as representative seed oil is available; however, this calibration does not take into consideration the effect of the seed matrix on the NMR signal of the oil, and therefore may not be entirely accurate. Also, the stability of extracted oil may be somewhat more poor than in the intact oilseed. Freshly extracted oil should be used to ensure a representative sample.

Secondary calibration using oilseeds of known oil content has the advantage that the matrix effect is accounted for in the offset of a linear X-Y correlation of observed NMR signal/gram versus known oil content by a reference method. Disadvantages include the difficulty of obtaining a series of oilseed samples with a suitable range of oil content to generate a calibration line, and the fact that the accuracy of the chosen reference method still limits the accuracy of the secondary calibration line. Precision of the NMR method is the same regardless of calibration method, as in the case of moisture measurements.

Instrument Considerations

It is necessary to use a benchtop NMR instrument that is suited for the necessary sample volume. If limited seed quantity is typical (e.g., in breeding programs when single seeds or seeds from a single plant or pod should be measured), it is advisable to use an instrument with the highest NMR frequency possible, that can still accommodate the sample, to ensure optimum signal-to-noise. When screening bulk seeds by NMR, sample quantity is generally not limited. In this case, the sample size should be as large as possible to ensure a representative sample, yet not so large as to cause poor NMR amplitude uniformity across the filling volume and from top to bottom. In the case of sunflower measurements, the size recommended by the USDA GIPSA (1999) is 50-mm diameter tubes filled to a maximum of 6 cm; therefore, the NMR instrument must be able to accommodate 50-mm tubes. For other large oilseeds (e.g., soybean or corn), typical sample tube size is 40-mm diameter or greater. Small oilseeds (e.g., canola, flax, cottonseed) can be measured in 18-mm diameter tubes or greater.

SIMULTANEOUS MOISTURE AND OIL DETERMINATION IN OIL SEEDS BY NMR

BASIC PROTOCOL

This rapid method for the determination of moisture and oil contents uses a benchtop pulsed NMR spectrometer and the Hahn echo pulse sequence (Rance and Byrd, 1983; Davis, 1989). A test sample is inserted into the static magnetic field of the spectrometer. Interaction of the field with the hydrogen in the sample causes a small magnetization to develop in the sample. The instrument reads the magnetization by a series of timed events. First, the sample is exposed to a short intense radio frequency (RF) pulse, which excites the hydrogen nuclei. The duration of the pulse is suitable to cause a 90° degree rotation of the sample's magnetization into the plane orthogonal to the static magnetic field (known as the X-Y plane). Next the instrument records the NMR signal (known as a Free Induction Decay or FID) in the X-Y plane for a period of milliseconds. During this time, some parts of the sample undergo NMR relaxation and return to the original state. The signal amplitude at 50 to 60 microseconds following the first RF pulse is proportional to the total hydrogen from moisture and oil components of the samples. At 3.5 milliseconds, a 180° RF pulse is applied, followed by a second period when the instrument records the NMR signal. The 180° RF pulse causes a refocusing of remaining X-Y magnetization, producing an echo signal at 7 msec (2×3.5 msec). The amplitude of the echo optimized in this way is proportional to the oil phase, hence the oil content of the sample. The difference between the amplitude recorded following the 90° RF pulse and the amplitude recorded at the spin-echo is proportional to the moisture content.

Materials

Uniform seed sample from a well-mixed lot, dry
Benchtop pulsed NMR analyzer (e.g., Bruker minispec) with sample compartment designed for absolute measurements
Sample tubes and caps
Balance with accuracy of at least the expected sample weight divided by 1,000 (optional)
Kimwipes
Temperature conditioning block (optional; see Critical Parameters)

Additional equipment and reagents for reference method (e.g., *UNIT A1.1* or *UNIT A1.2*).

Generate calibration standards and reference data

1. Obtain a uniform seed sample from a well-mixed lot and split it into parts, so that one part can be used for moisture determination by the reference method (the reference percent moisture) and the other part for NMR calibration.

   In order to obtain reference samples that have an appropriate range of known moisture and oil contents, it is first necessary to select the seed. It may be necessary to blend seed of high- and low-moisture content in order to obtain a suitable range for the calibration line. Samples must not be physically wet (see Strategic Planning).

   If calibration of percent oil by a secondary method is to be used (see Strategic Planning), then an additional part is needed for oil determination by the reference method (the reference percent oil).

   When sample quantity is absolutely limited, the NMR measurements can be made first, followed by moisture determination by the reference method on one part of the sample and oil determination on the other. Later, the reference moisture and oil values can be entered into the calibration file for correlating NMR signal/gram to the observed moisture and oil by the reference method.

2. Use the reference method (e.g., *UNIT A1.1* or *UNIT A1.2*) to determine the moisture content of each reference sample. Label each corresponding NMR reference sample from the split lot with the values to be used as reference values.

Calibrate the NMR instrument

3. Prepare the NMR instrument for measurements by equilibrating to stable operating conditions and tuning to correct 90° and 180° pulse lengths and resonance condition using the sample and procedure recommended by the manufacturer.

   The NMR analyzer should have a sample compartment that provides a uniform NMR amplitude response throughout the normal filling height and a sample capacity that is appropriate for the oilseed size.

4. From the user interface of the benchtop NMR instrument, load the application for simultaneous determination of moisture and oil in oilseeds.

   This may take various forms, depending on the instrument type and software version; therefore, consult the manufacturer.

5. Prepare the spin echo application with appropriate acquisition parameters. Typically, 90° to 180° pulse separation: 3.5 msec, moisture plus oil sample window: 0.05 to 0.06 msec, oil sample window: 7 msec, number of scans: 16, recycle delay: 2 sec, detection mode: "magnitude", and phase cycling (if available): "ON".

Calibrate for moisture

6. Prepare a reference sample of known weight for NMR measurement by placing the empty sample tube on a balance, taring, and then adding sample to the recommended filling height. Record the sample mass and immediately cap the tube to seal from atmospheric moisture. Label and repeat for all samples.

7. Once all samples are prepared, select the one with the highest combined moisture plus oil content to set the gain on the NMR instrument. Be sure to "save" the final parameters into the application.

8. Measure each sample using the "Calibrate" function. For each reference sample, enter the requested sample mass (step 6) in order to normalize the NMR signal. Enter the requested assigned moisture content (i.e., reference percent moisture; see step 2) for each sample.

9. After the last sample has been measured, terminate the calibrate measurement loop and examine the calibration data. Continue on to the oil calibration routine by accepting the moisture calibration data.

   Correlation between NMR signal/gram and reference percent moisture is normally near 0.98.

Calibrate for oil by primary method

10a. Prepare a sample from typical oilseed (as if it was to be used for NMR measurement), by filling a sample tube to the recommended filling height. Weigh the sample and record this weight as the reference mass.

   The recommended filling height may be 3 to 6 cm, depending on the NMR instrument specifications.

   The reference mass is assumed to be the typical weight of all oil standards.

11a. Place dry Kimwipe(s) in each tube to serve as an absorbent surface. Tare the balance with the tube plus Kimwipe.

   The Kimwipes should occupy approximately the entire filling height as discussed above, but not over.

12a. Using a Pasteur pipette, drop a small amount of oil (e.g., if the reference mass was 20 g, use 0.100 g) onto the Kimwipe, being careful not to get it on the wall of the tube above the Kimwipe. Record the weight to four significant digits.

The oil should be absorbed into the paper thereby distributing the oil in the measuring volume.

This sample represents the nearly zero sample (i.e., virtually no oil), which is used to establish an accurate intercept.

13a. Prepare 3 or more samples with increasing oil weights so that sample mass/reference mass × 100 is a percentage (the prepared percent oil) that spans the range of interest and extends to nearly zero oil.

As an example, for canola the percent oil should be from 60% to nearly zero.

14a. Measure each sample using the "Calibrate" function following the completion of the moisture calibration (from step 10). For each sample, enter the reference mass when weight is requested in order to normalize the NMR signal. Enter the prepared percent oil when the assigned oil content is requested. After the last sample has been measured, terminate the calibrate measurement loop and examine the calibration data. Complete the calibrate routine by accepting the calibration data, so that the data is saved to the hard disk.

Correlation between NMR signal/gram and prepared percent oil is normally near 1 (e.g., 0.999).

Calibrate oil content by secondary method

10b. Use the reference method to determine the oil content of each reference sample (i.e., reference percent oil). Label each corresponding NMR reference sample from the split lot with the values to be used as reference values.

Each reference sample should have a moisture and oil determination on the same or a split sample used for NMR.

NOTE: *If all reference samples used in the moisture calibration (steps 6 to 9) also have known oil content spanning an appropriate range, there is no need to prepare more samples for the oil calibration; however, it may be desirable to extend the oil calibration by use of additional oil samples that have well-known oil content, even if these same samples do not have known moisture values. If this is the case, prepare the extra oil reference samples in the same way as in step 6 above.*

11b. Measure each sample using the "Calibrate" function. Enter the actual sample mass (step 7) when requested in order to normalize the NMR signal. Also enter the requested assigned oil content (i.e., the reference percent oil).

12b. After the last sample has been measured, terminate the calibration measurement loop and examine the oil calibration data.

13b. Complete the calibration routine by accepting the oil calibration data.

14b. Save the moisture and oil calibration data to the hard disk.

Correlation between NMR signal/gram and reference percent oil is normally near 0.98.

Measurements of unknown samples

15. *For samples with higher moisture than normal preservation level (optional):* Dry the samples at 103°C for 1 hr prior to the NMR analysis of the dried sample.

Examples of higher moisture than normal preservation level: >10% for canola and sunflower, >14% for soybean. Total water content is the residual moisture obtained by

NMR plus the moisture loss upon drying. Please refer to details in ISO 10565 (1999) for more details.

All moisture must be in association with starches and other solids in the matrix in order for the standard NMR measurement to be applicable. As in the case of calibration, samples must not be physically wet.

16. Obtain a uniform seed sample from a well-mixed lot.

    Reduction of the sample from bulk to a size that can be measured by NMR must be done according to the appropriate method for the sample of interest.

17. Place the empty sample tube on a balance and tare. Add sample to the recommended filling height and record the actual sample mass. Cap tightly to seal the sample from atmospheric moisture.

18. Measure each sample using the "Measure" routine. For all samples, enter the actual sample mass as determined above when requested in order to normalize the NMR signal.

    Use the spin echo application that was previously prepared and calibrated for moisture and oil with appropriate parameters. Instrument parameters must remain exactly the same as used at the time of calibration.

COMMENTARY

Background Information

Agricultural products are often subjected to dehydration to improve storage stability; grains, and seeds are typical examples. Critical moisture contents must be achieved so that these products can be stored safely. This is closely related to the economic value of crops such as peanuts where size, weight, and presence of aflatoxin are of critical importance. Accurate measurement of moisture content is important to effect an efficient drying system as a part of quality control. Furthermore, moisture can affect the processing of the grains. Wheat for instance must be tempered to critical

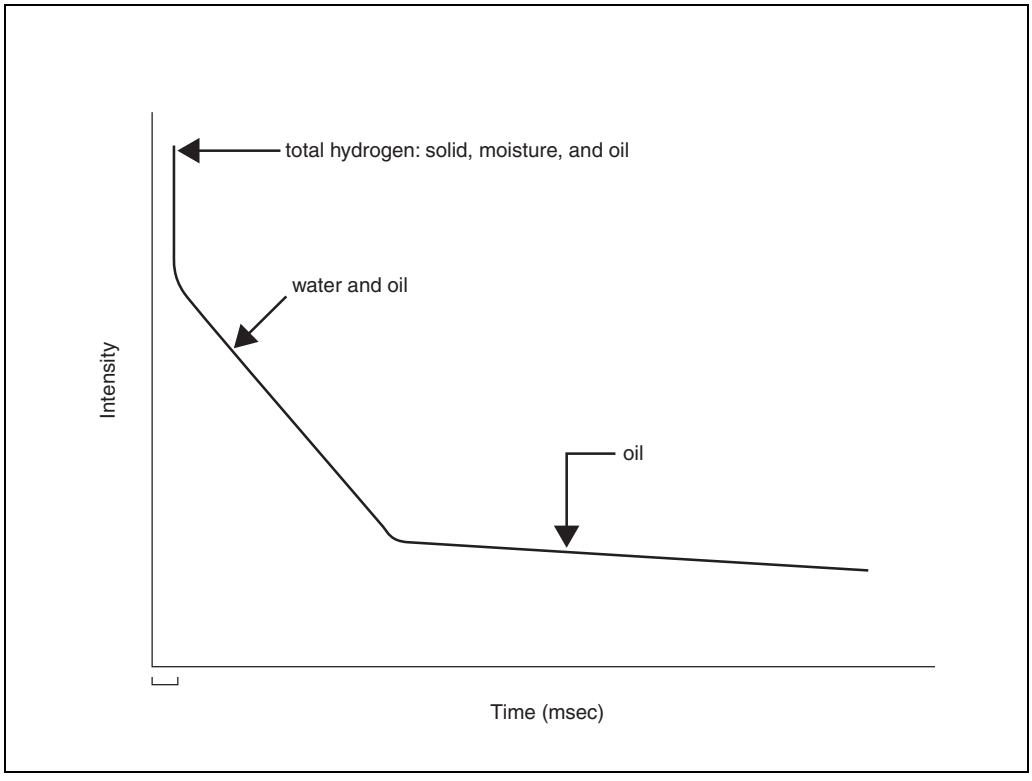

Figure A1.3.1 Free Induction Decay (FID) after a 90° pulse showing a three-component system.

moisture content before it can be properly milled and rice-hulling losses are at the minimum when hulled at 15% moisture content. In oil seeds, an accurate measurement of the seed moisture content is important to the economic value since oil content determination requires knowledge of the moisture content. Rapid methods for moisture and oil determination of all commercial oilseeds are necessary to assess the quality of a large pool of oilseeds available globally.

Pulsed nuclear magnetic resonance (NMR) has been used for such analyses due to the speed, repeatability, and nondestructive nature of the measurement. Additionally, the method requires no solvents, an important consideration for environment friendliness. Modern benchtop NMR systems are highly automated and computerized, which allows unskilled personnel to be trained to run the measurements.

This procedure is based on the unique response of the hydrogen nuclei (protons) present in bound moisture versus free oil. The physical state of the moisture and oil is a critical factor in experiment design. Since the oil in typical oilseeds is present in a liquid state and moisture is bound under conditions in a mature seed, a simple spin echo pulse sequence can determine the quantities separately.

In the current method, the signal intensity of the hydrogen nuclei present in the entire sample is recorded at particular times in the NMR signal decay, and is expressed per unit sample weight. The free induction decay (FID) has a maximum intensity when all the spins of the hydrogen nuclei have been rotated 90° respective to the permanent magnetic field (B_o). This intensity is directly proportional to the total number of hydrogen nuclei present (Fig. A1.3.1). Hydrogen nuclei in different physical or chemical environments decay at different rates and thus the resulting FID initially has the contribution from solids, bound moisture, and oil. During the first 0.05 msec following the 90° pulse, the signal contributed from the solids decays to zero and only the signals from moisture and oil components remain.

In this procedure, the signal generated during a Hahn Echo (also called Spin Echo) pulse sequence is analyzed rather than the signal following a simple 90° pulse. A schematic drawing of the experiment is provided in Figure A1.3.2, and an actual example of the NMR signal from oilseed is given in Figure A1.3.3. The experiment begins with a 90° radio frequency pulse (as indicated by a narrow rectangle in Figure A1.3.2) to produce X-Y magnetization, followed by an initial data sampling period over a time interval called τ, after which

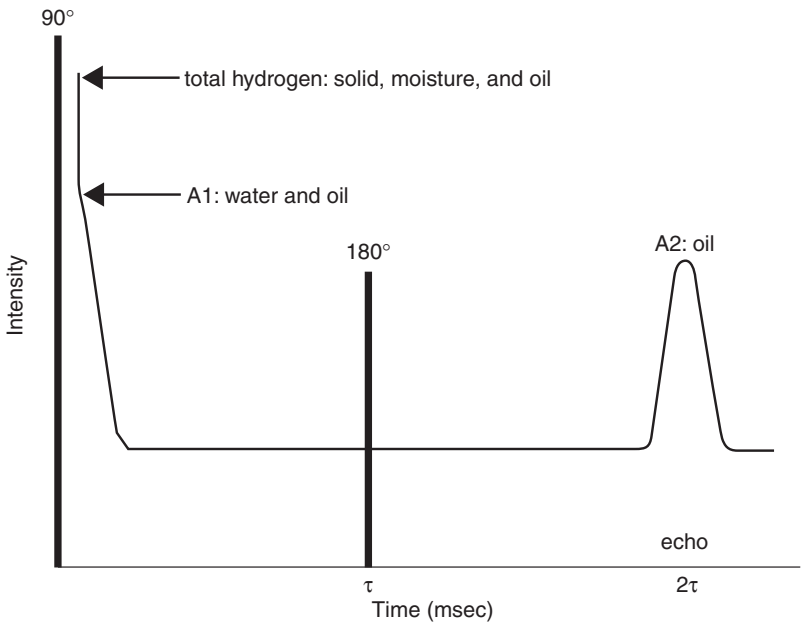

Figure A1.3.2 Hahn Echo (Spin Echo) pulse sequence and schematic NMR signal from an oilseed. Label A1 indicates the position of the water plus oil sampling window. The label A2 indicates the oil-only sampling window.

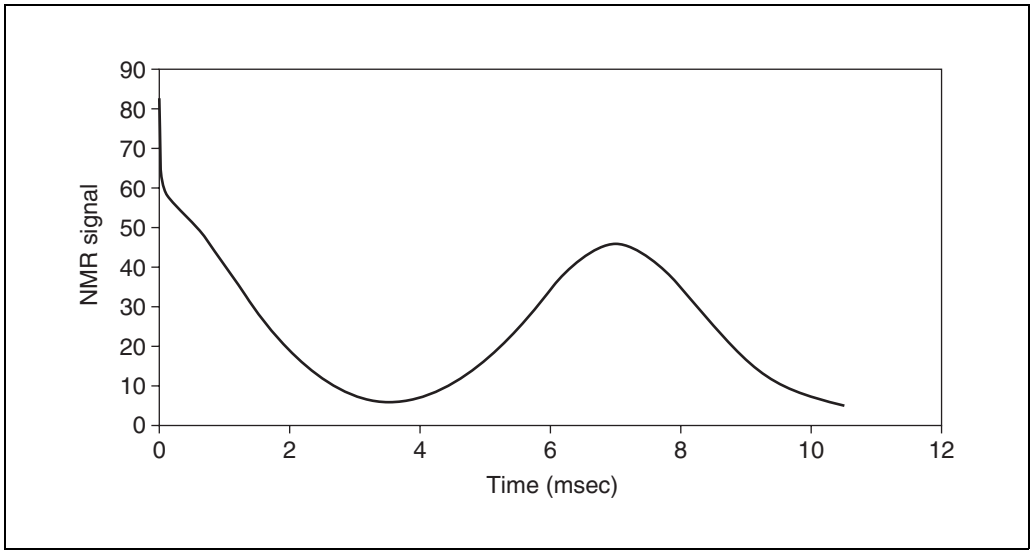

Figure A1.3.3 Hahn Echo NMR signal of canola at 20 MHz obtained by a minispec mq20.

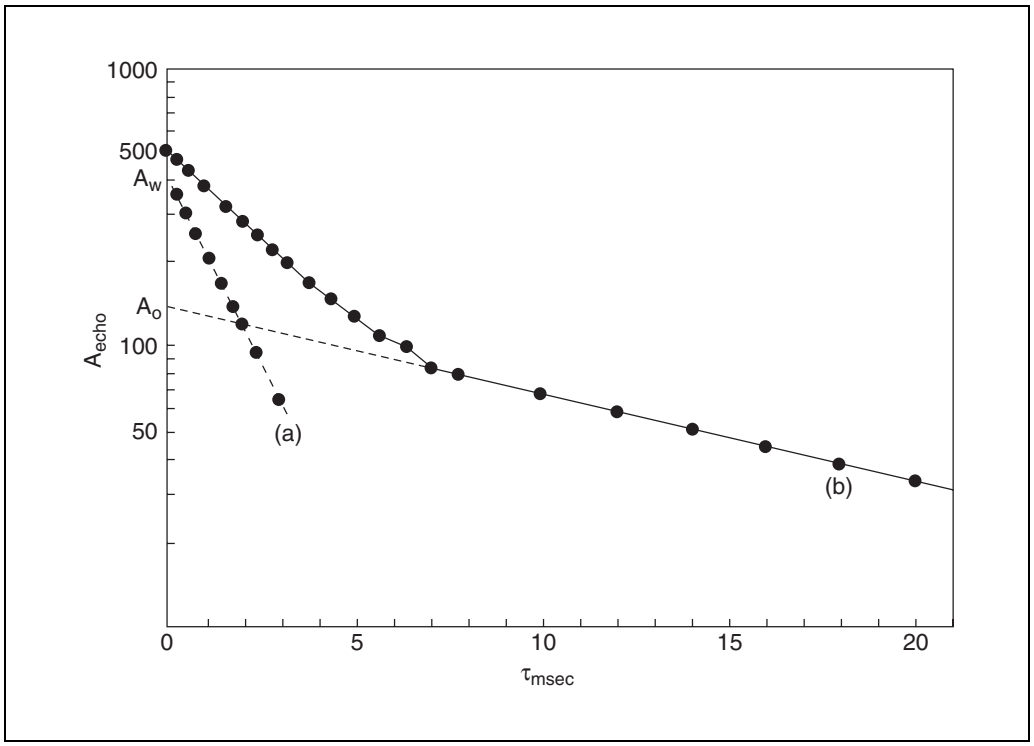

Figure A1.3.4 Semi-log plot of Spin Echo amplitude with changing Hahn Echo experiment interval, τ, for an oilseed containing excess moisture (therefore some is free) and oil. Extrapolation of the fitted lines (dashed) to $\tau = 0$ gives amplitudes for excess (free) moisture (A_w and oil A_o; modified from Schmidt, 1991).

a 180° radio frequency pulse is used to cause a refocusing of the remaining X-Y magnetization, and a second data sampling period records the resulting "echo" over an interval twice as long (therefore equal to 2τ) as the first data sampling period. The interval τ must be long enough to allow the moisture signal to decay to zero intensity prior to the 180° refocusing pulse. In this method τ is set to 3.5 msec. This refocusing part of the experiment is useful for recovering the entire remaining oil signal at the echo independent of the signal from solids and bound moisture. From the data, two signal amplitudes are evaluated and normalized by sample weight. The signal amplitude per gram from 50 to 60 msec following the 90° pulse is proportional to the moisture plus oil content of the sample, while the amplitude per gram at the peak of the echo (at a time corresponding to 2τ where τ is 3.5 msec) is proportional to the oil content of the sample. Note that for the first evaluated window from 50 to 60 μsec, the moisture signal amplitude is calculated by subtracting the NMR signal amplitude contributed by oil, and the difference, normalized by weight, is proportional to percent moisture.

The methodology described in this procedure is limited to systems that do not contain free moisture. In cases when both oil and water signals are refocused after the second pulse (e.g., when free water is present), the spin-echo decay curve can be collected over varying τ values. Log of the echo amplitudes versus τ can be further analyzed to deconvolute the contribution of various components, in this example water and oil would result in a biexponential decay curve. Extrapolating each decay curve to $\tau = 0$ gives the signals amplitudes due to the oil and the water fractions (A_o and A_w; Fig. A1.3.4).

Critical Parameters

Sample temperature can be important for two reasons. If the oil is not completely liquid at the measurement temperature, then a lower NMR signal/gram will be obtained than is normal; therefore, to avoid errors due to the state of oil, samples should be measured at a temperature above the melting point of the oil.

Table A1.3.1 Typical Results for Percent Moisture in Soybean by Benchtop NMR and Comparison to Reference Method Values

Sample	1	2	3
Analyzed weight (g)	21.4200	21.2784	21.6552
Trial 1 moisture[a] (%)	10.64	12.33	10.87
Trial 2 moisture[a] (%)	10.67	12.38	10.87
Trial 3 moisture[a] (%)	10.67	12.34	10.86
NMR average (%)	10.66	12.35	10.87
Given moisture[b]	10.45 ± –0.01	12.29 ± –0.03	11.16 ± 0.14
Std dev	0.0173	0.0265	0.0058
Relative std dev (%)	0.162	0.214	0.053

[a]Determined by NMR.
[b]Determined by weight loss upon drying.

Table A1.3.2 Typical Percent Oil in Soybean by Benchtop NMR and Comparison to Reference Method Values

Sample	1	2	3
Analyzed weight (g)	21.4200	21.2784	21.6552
Trial 1 oil[a] (%)	20.01	19.66	20.07
Trial 2 oil[a] (%)	19.99	19.68	20.08
Trial 3 oil[a] (%)	20.03	19.69	20.09
NMR Average %	20.01	19.68	20.08
Given oil (%)	19.86 ± 0/07	19.04 ± 0.11	19.82 ± 0.14
Std dev	0.200	0.0153	0.0100
Relative std dev (%)	0.100	0.078	0.050

[a]Determined by NMR.

Table A1.3.3 Soybean Sampling Variation[a]

Sample no.[a]	Average oil[b] (%)	Average moisture[b] (%)
Sample 1A	20.10	10.66
Sample 1B	10.96	10.57
Sample 2A	19.68	12.35
Sample 2B	19.86	12.22
Sample 3A	20.08	10.87
Sample 3B	19.99	10.81

[a]A and B refer to two different NMR samples taken from the same soybean type.
[b]Average is determined from three different measurements on the same soybean NMR sample.

In addition, NMR signal amplitude for all parts of the sample (moisture, oil, and other) depends on temperature due to the effect temperature has on the population differences (Boltzman's distribution) between the lower energy and higher energy spin states. As temperature increases, the population difference decreases, causing less of an energy separation between allowed nuclear spin states, and therefore less NMR signal per hydrogen nucleus. If room temperature varies significantly (e.g., by more than 2°C), samples should be conditioned to a standard temperature in an aluminum block prior to measurement or a number of calibration lines can be prepared at intervals of 2°C, so the correct line can be selected to suit the temperature at the time of actual measurement.

The reproducibility is improved if test portions are maintained at a constant volume. It is recommended to consistently check the calibration for moisture and oil contents against standards.

Anticipated Results

Typical moisture content results for soybean samples are shown in Table A1.3.1. Three samples were prepared and each was measured three times in the same minispec analyzer. The NMR percentage moisture was found to be very reproducible (standard deviation for three measurements was typically better than 0.03) and agreement of NMR with the reference method was typically ±0.15% moisture or better.

Typical oil content results for soybean samples are shown in Table A1.3.2. In this case three samples were prepared and each was measured three times in the same minispec analyzer. The NMR percentage oil was again found to be very reproducible (standard deviation for three measurements was typically 0.02 and better) and agreement of NMR with the reference method was typically ±0.3% oil or better.

Subsampling variation for soybean samples were found to be ±0.1% (for oil and moisture) as shown in Table A1.3.3.

Time Considerations

The benchtop NMR instrument should be turned on for a reasonable time (2 to 3 hr) before sample analysis. Actual analysis takes only a few minutes, but the instrument should be at equilibrium. It is a common practice to have a standard sample to occasionally test the general performance of the instrument.

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Traditional Indirect Methods for Estimation of Water Content: Measurement of °Brix

Most measures of water content are indirect, estimating water content as the difference between the total mass of a sample and the mass of the same sample in the absence of water. Two primary strategies exist. The first is to perform actual weight determinations upon the sample—i.e., before and after drying (*UNIT A1.1*). Here the assumption is that the only material lost in the drying process is water. The second strategy is to somehow determine the concentration of solute within the sample. Note that two assumptions have to be made in order to estimate water content. First, it must be assumed that the property measured has a repeatable dependence upon the solute concentration, stated in appropriate units. Second, it must be assumed that this concentration, in such units, can be accurately converted to a concentration stated on a weight basis. It is important, when considering the use of any indirect determination of water content, to be aware of these inherent assumptions, and to consider to what extent these assumptions will hold in the real system of interest.

The use of drying as a method of determining water content has been described in *UNIT A1.1*. In the current discussion, indirect methods which depend upon the estimation of solute concentration will be considered. This estimation will utilize some form of calibration curve which correlates a measured property with the concentration. Many traditional methods exist, since water content—or solute (solids) content—is an important measure to describe the state of a system. It is a particularly important measure in commerce, where either the solute content or the water content may be an important indicator of quality or value.

Two long-established methods for the estimation of solute content are based upon the observations that both the solution density and the refractive index of a solution change as the solute concentration of the solution changes. The methods that have been developed for estimating solution concentrations based upon these properties, and the underlying assumptions and limitations of these methods, can be best demonstrated by looking separately at how each method can be used to estimate the sugar content of solutions, and hence, by difference, their moisture content.

In both cases, an appropriate concentration unit to characterize the solution is termed °Brix. This is a measure of concentration equivalent to grams sucrose per 100 grams solution. Hence, to describe a solution as having a concentration of 10 °Brix means that it is a solution in which 10 g sucrose are contained in 100 g solution. Assuming the solvent to be water and the solute to be sucrose, the water content of 100 g of a solution is (100 – °Brix).

Correlation curves can be drawn by plotting either solution density or refractive index against °Brix. The accurate estimation of solution water content would appear to be a trivial second step. However, this is so only if the solute is pure sucrose. Also, since both density and refractive index are dependent upon temperature, the temperature of measurement must be known, and a calibration curve valid for that temperature must be available.

Solutes other than sucrose also cause there to be both density and refractive index changes as their concentrations increase. These changes can be interpreted as reflecting solute concentrations expressed in °Brix; however, this concentration scale now becomes a scale that indicates the concentration of a sucrose solution which has the same density (or refractive index) as that of the unknown solution. Only in the case where the weight concentration of the solute in this unknown is the same as the weight concentration of the equivalent sucrose solution can subtraction give a reliable estimate of water content. Nonetheless, °Brix still gives a useful comparative scale for solutions where the composition of the solute is unchanged in the different solutions. Alternatively, if a correlation exists between apparent °Brix for the solution of interest and the actual percent-weight concentration of that solution, an estimate of both solids and water content can be made.

Bearing in mind, from the discussion above, that °Brix really only allows estimation of relative water content in a series of related systems, this measure is used sufficiently frequently to require that the procedures be outlined. The sections below describe the use of the refractometer and the hydrometer for determining water concentration. These instruments are readily available from a number of sources including Brannan and ERTCO Precision (for

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hydrometers), ATAGO, MISCO, Reichert, and Topac (for refractometers), or general suppliers such as Fisher and Cole Parmer.

CONCENTRATION ESTIMATION BY REFRACTOMETER

Refractive index is a consequence of the changing velocity of light in different media. It is equal to the ratio of the wave velocity of light in a vacuum to the wave velocity of light in a specific medium. If light in a vacuum is incident upon a plane surface boundary with the medium, the index of refraction is also the ratio of the sine of the angle of incidence of the ray of light in a vacuum to the sine of the angle of refraction in the medium.

As a practical matter, air is usually used as the reference medium rather than a vacuum. As the index of refraction of air at 1 atmosphere pressure and 25°C is 1.00027, this value can be used as a multiplier to arrive at the true index of refraction of the system.

The refractive index is a physical property of the medium that depends upon the wavelength of the light and the temperature of measurement. It can be determined by identifying the critical angle of reflection, which is the angle of incidence to a boundary surface of a light ray within the medium at which the angle of refraction is 90°. Any light ray within the medium incident upon the surface with an angle of incidence greater than the critical angle will experience total internal reflection.

In practice, the critical angle of reflection is measured at the boundary of a liquid with a glass prism of known refractive index, rather than at the liquid/air boundary. A commonly available instrument is the Abbe Refractometer, which, by use of compensating prisms known as Amici prisms, allows the refractive index for light of the wavelength of the sodium D line to be measured using white light. This is convenient, as a source of white light is more readily available than is a sodium D source, and hence portable refractometers are feasible.

The basic method is to use a manual Abbe refractometer to determine refractive index. Various automated or electronic instruments exist which automatically perform some of the steps of the manual procedure. The first requirement is that the sample be a solution. In some instruments, the solution is placed between two prisms, and the image of the critical ray boundary is adjusted to meet a reference mark; for this adjustment, the refractive index and equivalent °Brix can be read from a scale. The sample temperature must be known, or the instrument must have temperature compensation. Some automatic digital refractometers use the same methodology of sample presentation, but automate the matching of the critical boundary to the reference marker.

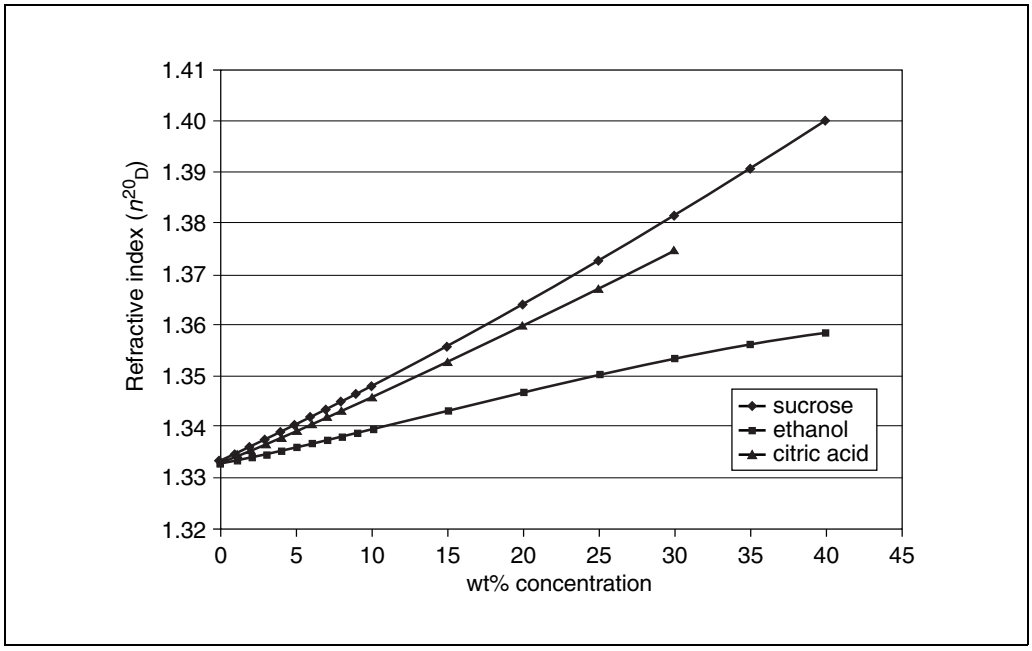

Figure A1.4.1 Concentration dependence of refractive indices of sucrose, ethanol, and citric acid solutions at 20°C. Data based on tables in *Handbook of Chemistry and Physics, 61st Edition* (Weast, 1980).

Other automatic instruments do not require the sample to be inserted between two prisms. These require the sample to be placed on a glass surface, and detection is of the critical reflection internal to the glass. In this type of instrument, the detection is electronic, and temperature compensation can be programmed into the operational procedure.

The type of refractometer used depends upon the precision needed, and also the resources available. A simple hand-held refractometer provides adequate results for quality control in the field. A more complex manual instrument provides good data, but throughput can be limited. Electronic instruments provide a rapid method and a wide range of sensitivities.

In all cases, the initial measurement is of a refractive index which is correlated to the refractive indices of a series of standard solutions of known concentration, with °Brix being the most common standard set. Figures A1.4.1 and A1.4.2 illustrate the dependence of refractive index on concentration for a range of solutions.

CONCENTRATION ESTIMATION BY HYDROMETER

A hydrometer is used to determine the density or specific gravity of a liquid using the principles of buoyancy. Buoyancy results from the upthrust on any object immersed or partially immersed in a liquid. Upthrust is developed as a consequence of the weight of the displaced liquid. At the point of partial immersion at which this upthrust just equals the weight of the hydrometer, the hydrometer will float. The liquid level can be measured against a scale etched on the hydrometer. The density is simply the weight of the hydrometer divided by the volume of liquid displaced as it floats.

The hydrometer is designed with a wide bulbous base and a narrow tube-shaped top. Assuming that the liquid level is within the tube section, the volume of liquid displaced is that of the bulb plus that equivalent to the position of the liquid level on the stem. As the bulb volume is large in contrast to the stem, a small change in density will result in a large shift in level on the stem. The levels corresponding to different displaced volumes (and hence densities) are marked on the stem. These levels can be labeled as density values or as equivalent sugar solution concentrations (°Brix). The relative sizes of the bulb and stem determine the density/specific gravity range for the hydrometer.

Hydrometers are precalibrated by the manufacturer. The calibrations are valid only for a particular temperature. It is prudent to verify the calibration using known sugar solutions. Different hydrometers vary in the ratio of main bulb volume to measuring stem volume and also differ in mass. Hence the immersion level is in the stem region for different density/specific gravity ranges. Also, the sensitivity is increased by having a relatively thin stem, but at the same time this decreases the range.

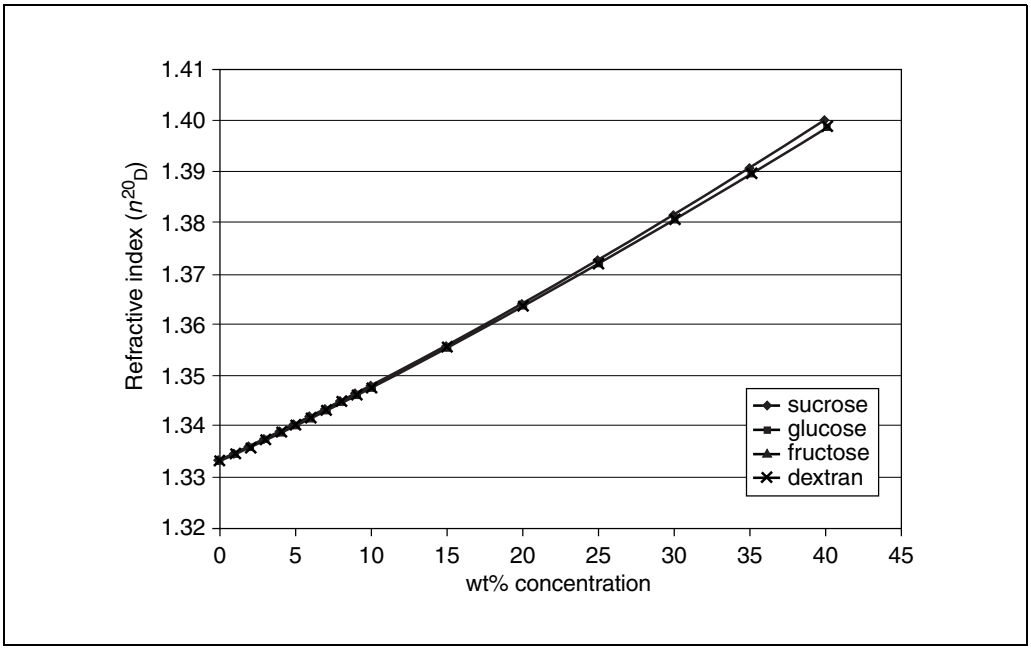

Figure A1.4.2 Concentration dependence of refractive indices of carbohydrate solutions at 20°C. Data based on tables in *Handbook of Chemistry and Physics, 61st Edition* (Weast, 1980).

To use a hydrometer, simply float it in the solution to be evaluated and note the level. Temperature control is imperative. Also, the hydrometer must be cleaned and dried prior to immersion, so as to avoid contamination of the sample.

INHERENT ERRORS IN HYDROMETERY AND REFRACTROMETRY FOR WATER ESTIMATION

Given that "equivalent °Brix" can readily be determined, how reliable are interpretations in terms of water content? Figures A1.4.1 through A1.4.4 show the relationship between solution concentration (percent weight; wt%) and either

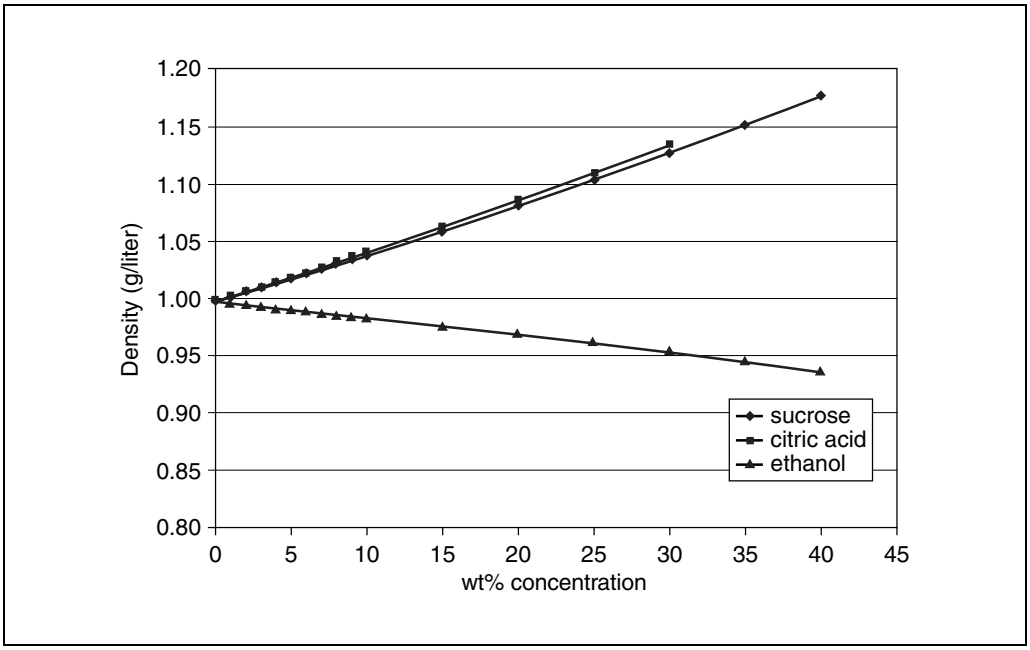

Figure A1.4.3 Concentration dependence of densities of sucrose, citric acid, and ethanol solutions at 20°C. Data based on tables in *Handbook of Chemistry and Physics, 61st Edition* (Weast, 1980).

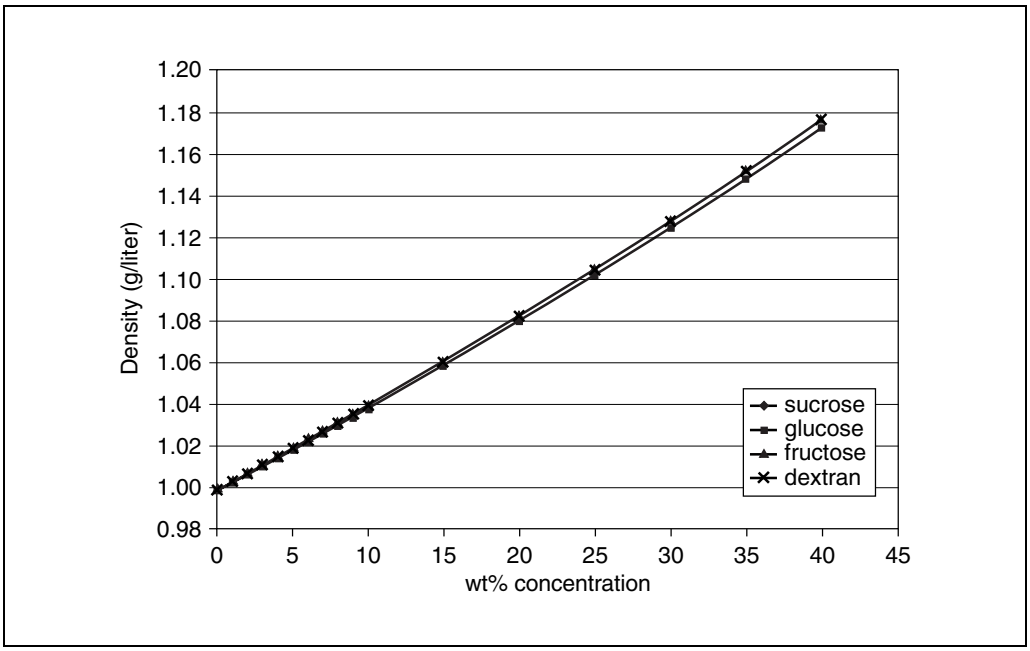

Figure A1.4.4 Concentration dependence of densities of carbohydrate solutions at 20°C. Data based on tables in *Handbook of Chemistry and Physics, 61st Edition* (Weast, 1980).

refractive index or density for several solutes. By examination, if a solution is made of sucrose, a °Brix reading using a refractometer will be correct. If it is citric acid, however, a °Brix reading will underestimate the true percent-weight concentration. Solutions of glucose, fructose, or dextran will also give a smaller °Brix reading than the true concentration. For many purposes, the errors are sufficiently small to be ignored. If more precision is needed, a calibration of refractive index against actual percent-weight concentration for the particular composition of solute must be performed. For fruit juices, if the acid concentration is known, the °Brix reading can be adjusted by assuming that each 1.2 wt% acid causes a °Brix contribution of 1.0. By subtraction from the measured °Brix, the sugar content in °Brix can then be estimated.

Similar comments apply to the apparent °Brix and true percent-weight concentrations determined by density/specific gravity measurement. Assuming the percent-weight concentration is accurate, for a concentration of x (wt%), moisture content is $(100 - x)$%.

Clearly, the calculation of moisture content by difference, whether using hydrometry or refractometry to obtain estimates of solute concentration, is an approximate method. Its utility depends upon there being a repeatable correlation between either density or refractive index and the true percent weight concentration of the solution. This repeatability is dependent upon the exact composition of the solute system.

In addition to the tables in the *Handbook of Chemistry and Physics* (Weast, 1980) used to construct Figures A1.4.1 to A1.4.4, many tables exist in the literature that pertain to specific products. For example, an appendix in Nagy et al. (1993) gives data for sucrose solutions, and table C1 in Downing (1989) gives data for a range of solutions.

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Factors to Consider When Estimating Water Vapor Pressure

Under equilibrium conditions, the partial vapor pressure of water above an aqueous sample is defined by thermodynamic parameters. The measurement of vapor pressure, therefore, is linked to thermodynamic principles, though in a real system, which may or may not be at equilibrium, the vapor pressure is not necessarily that which is to be expected at equilibrium. In order to select a methodology for determining vapor pressure or related properties, the limitations imposed by any requirement for equilibrium and the consequences of a departure from equilibrium must be understood. The purpose of this commentary is to clarify the generic constraints that exist when one attempts to determine the water vapor pressure of a sample.

As indicated above, the equilibrium partial vapor pressure of water defines its thermodynamic state. The thermodynamic state is usually described by use of the water activity concept, which defines water activity as:

$a_w = [p_w/p_w^0]_T$

where a_w is the water activity, p_w is the vapor pressure of water in equilibrium with the food sample, and p_w^0 is the vapor pressure of pure water at the same temperature, T. Water activity is related to other thermodynamic descriptors by standard relationships, which can be found in any chemistry textbook.

It is important to note the constraints imposed by the definition of activity. These are (1) that the system be at equilibrium and (2) that the comparison be an isothermal compari-

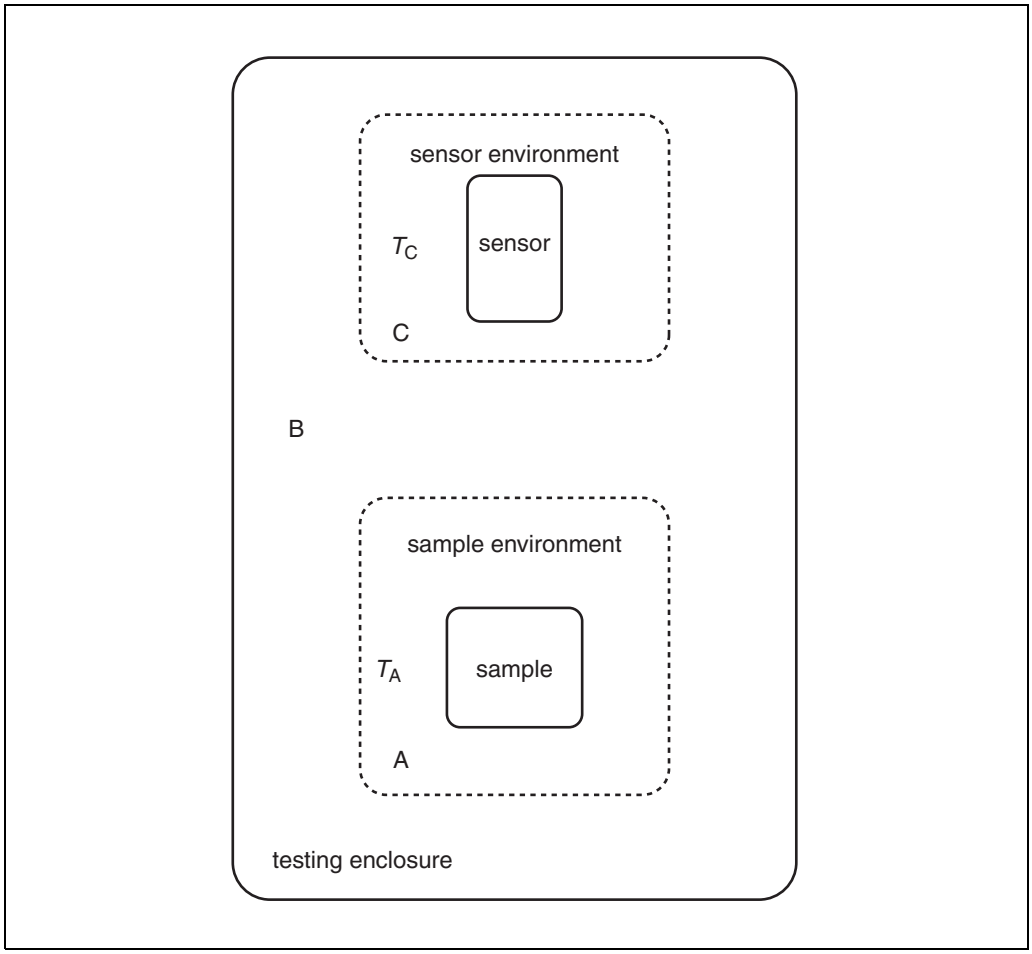

Figure A2.1.1 A representation of a vapor-pressure-evaluating system. Region A includes the sample and its environment. Region B is a vapor transfer path to the sensor region, C, which includes the sensor and its environment. T_A and T_C are the temperatures in regions A and C, respectively.

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son (i.e., the pure water reference is at the same temperature as the sample).

The consequences of these constraints can be considered when examining real measurement systems. The generic process for evaluation of p_w utilizes a system shown schematically in Figure A2.1.1. This depicts a measuring cell for the estimation of the partial pressure of water vapor in steady state with a sample. Region A consists of the sample and its immediate environment. Region B is a vapor transfer path to the sensor region. Region C is the sensor and its immediate environment.

The requirement for equilibrium should be considered first. It is necessary that region C be truly at equilibrium. Unless this region is at equilibrium, the relationship between the sensor and its immediate environment is not uniquely defined. Hence, for sensor measurements to be definitive, equilibrium must exist in region C. For region A, in a real measurement situation, equilibrium is not required to be established. It is sufficient that the p_w of the immediate environment (p_A) be in a steady state relationship with the sample, unchanging on a time scale defined by the investigator.

The temperature requirement should also be considered. Region C must be at a uniform, known temperature, T_C. This follows from the requirement for equilibrium. Region A should also be at a uniform, known temperature, T_A, because the state of the sample must be defined. It is not necessary that region A and region C be at the same temperature; indeed, in a dew-point cell they are at very different temperatures.

It is important that no part of the system be at a temperature below that of the dew point, which corresponds to p_w; should this be the case, condensation will occur, removing water from the atmosphere until the p_w of the system reaches a lower value that corresponds to a dew point equal to the lower temperature. When a measurement is taken, the partial pressure of water vapor should be uniform throughout the measuring chamber. In this case, p_w should be equal to p_A, p_B, and p_C.

The sensitivity of the sample or the sensor to vapor transfer must also be considered. Here, the quantity of material represented by the sample or by the sensor is important. Vapor pressure is established by the presence of a particular number of molecules in a defined volume of space. The transfer of water molecules into the vapor phase may cause a measurable change in the gravimetric water contents of the sample and sensor. It is necessary that the sample water content (or the initial sample weight) be known. It may also be necessary to

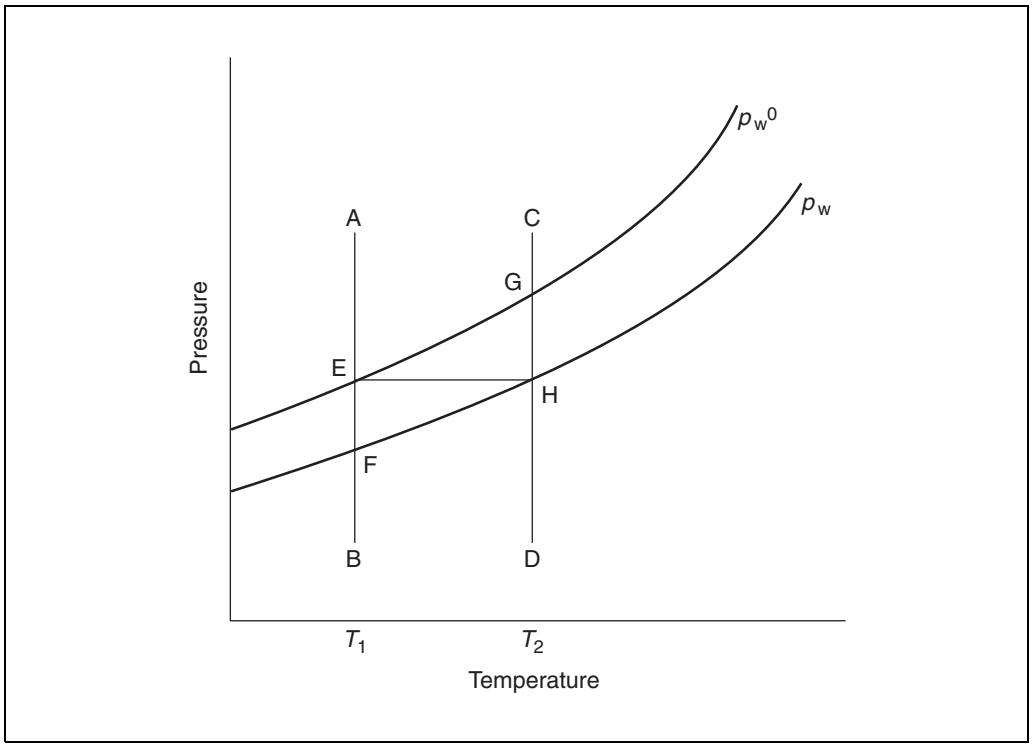

Figure A2.1.2 Effect of temperature on vapor pressure measurement. The upper curve is the vapor pressure of pure water, p_w^0. The lower curve is a system whose partial water vapor pressure, p_w, is always a constant fraction of the vapor pressure of pure water. See text for details.

know the sensor water content, depending upon the operating principle of the sensor.

In one operational mode, the system p_w is established by a large sample and is characterized by the sensor. The amount of moisture transfer from the sample does not significantly change its moisture content. The sensor is queried to identify its condition, and the result is converted into an estimation of p_w.

In another mode, the sensor becomes the controlling source that establishes the system p_w, and the condition of the sample is identified once it has reached steady state.

The influence of temperature on the measurement is best illustrated by referring to Figure A2.1.2. Here, the upper curve represents the vapor pressure of pure water, p_w^0, as a function of temperature, and the lower curve represents a system whose partial water vapor pressure is always a constant fraction, α, of the vapor pressure of pure water. The points E and G represent p_w^0 at temperatures T_1 and T_2, respectively. The points F and H represent p_w at temperatures T_1 and T_2, respectively. At both T_1 and T_2, the ratio of the vapor pressures $[p_w/p_w^0]_T$ is α (i.e., the ratios of the lines BF/BE and the lines DH/DG are both equal to α).

The line EH represents constant partial water vapor pressure, as should exist in the system of Figure A2.1.1. The point E at T_1 is the dew point for the sample with vapor pressure represented by the point H at T_2. Any point on the line EH and its extension to higher temperatures represents the uniform vapor pressure in the system. At different temperatures, the reference vapor pressure, $[p_w^0]_T$, is obtained from the upper curve. Clearly, though the partial vapor pressure, p_w, in the cell remains unchanged, $[p_w/p_w^0]_T$ depends upon the temperature, being 1 at T_1, α at T_2, and decreasing further as T increases.

Unless the temperature of the sample is known, the relative humidity in the sample region is unknown. Unless the sensor temperature is known, the relative humidity indicated by the sensor cannot be converted into a reliable estimate of the vapor pressure throughout the system. Without careful control and measurement of temperatures in the regions occupied by sample and by sensor, no meaningful data can be collected.

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This presents a good summary of the thermodynamic quantities interrelated to water activity.

Contributed by David Reid
University of California
Davis, California

Dew-Point Method for the Determination of Water Activity

The single most important property of water in food systems is water activity (a_W; Taoukis et al., 1988). Throughout history the importance of controlling water in food by drying, freezing, or addition of sugar or salt was recognized for preserving and controlling food quality. Water activity is a measure of the energy status of the water in a system (or the degree to which the water is "bound"), and hence of its availability to act as a solvent and participate in chemical or biochemical reactions and growth of microorganisms. It is an important property that can be used to predict the stability and safety of food with respect to microbial growth, rates of deteriorative reactions, and chemical or physical properties (Figure A2.2.1). The water activity principle has been incorporated by various regulatory agencies (e.g., Food and Drug Administration Code of Federal Regulations Title 21) in defining safety regulations regarding growth and proliferation of undesirable microorganisms, potentially hazardous foods, standards of several preserved foods, and packaging requirements (Fontana, 1998).

The Basic Protocol describes the determination of water activity of a product using a chilled mirror dew-point water activity meter. Dew point is a primary measurement of vapor pressure that has been in use for decades (Harris, 1995). Dew-point instruments are accurate, fast, simple to use, and precise (Richard and Labuza, 1990; Snavely et al., 1990; Roa and Tapia de Daza, 1991). In a dew-point instrument, water activity is measured by equilibrating the liquid-phase water in the food sample with the vapor-phase water in the headspace, and then measuring the vapor pressure of the headspace. The basic principle involved in dew-point determinations of vapor pressure in air is that air may be cooled without change in water content until it saturates. The dew-point temperature is the temperature at which the air reaches saturation. It is determined in practice by measuring

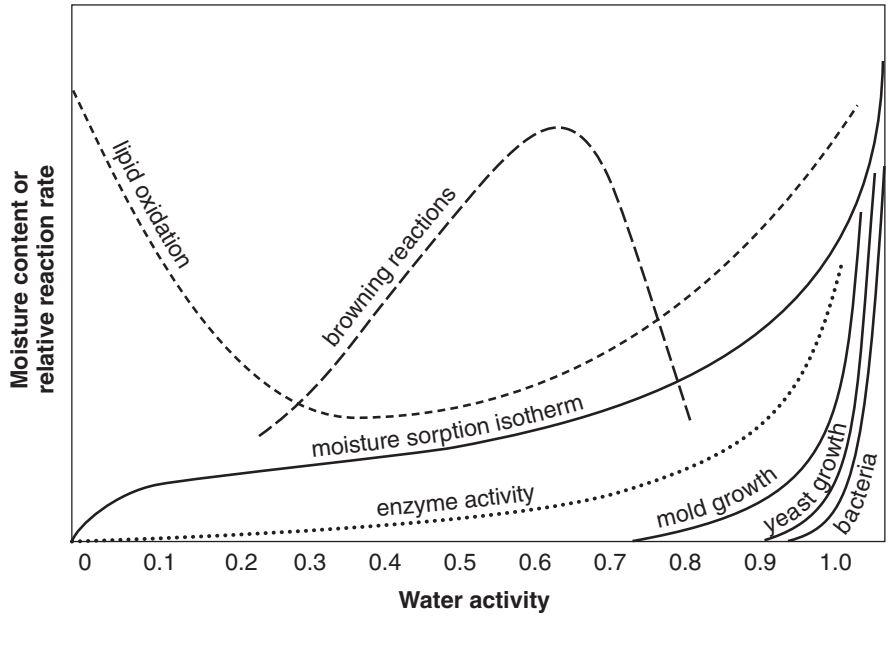

Figure A2.2.1 Water activity stability map (adapted form Labuza, 1970). A representation of a typical sorption isotherm for food materials and of the effects of water activity on the relative reaction rates of several chemical processes, as well as the growth of microorganisms, in foods are shown.

Contributed by Anthony J. Fontana
Current Protocols in Food Analytical Chemistry (2001) A2.2.1-A2.2.10

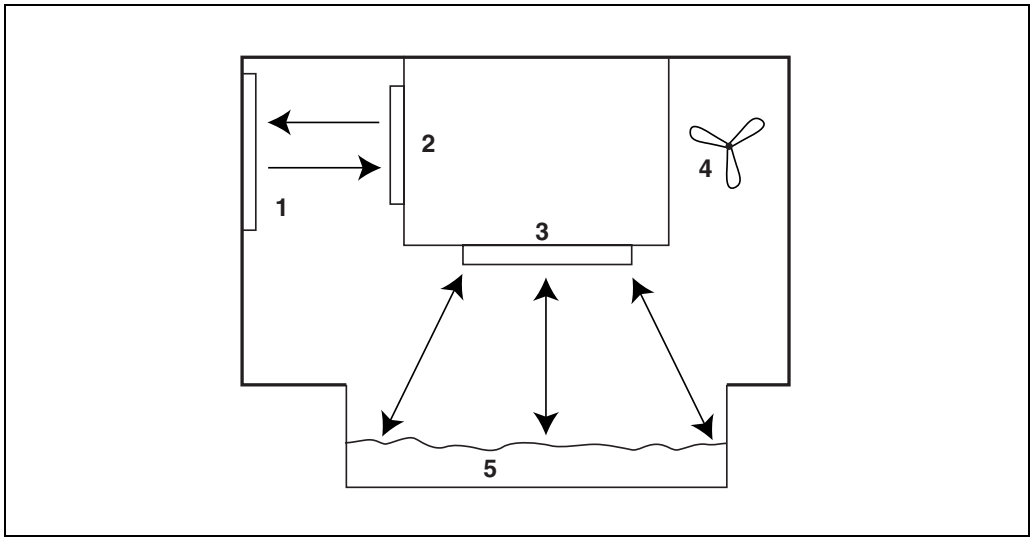

Figure A2.2.2 A representation of a modern dew-point chamber consisting of (1) mirror, (2) optical sensor, (3) infrared thermometer, (4) internal chamber fan, and (5) sample cup with sample.

the temperature of a chilled mirror when condensation starts. The water activity of the sample is the ratio of the saturation vapor pressure at dew-point temperature to the saturation vapor pressure at the sample temperature.

In a modern dew-point instrument, a sample is equilibrated within the headspace of a sealed chamber containing a mirror, an optical sensor, an internal fan, and an infrared thermometer (Figure A2.2.2). At equilibrium, the relative humidity of the air in the chamber is the same as the water activity of the sample. A thermoelectric (Peltier) cooler precisely controls the mirror temperature. An optical reflectance sensor detects the exact point at which condensation first appears; a beam of infrared light is directed onto the mirror and reflected back to a photodetector, which detects the change in reflectance when condensation occurs on the mirror. A thermocouple attached to the mirror accurately measures the dew-point temperature. The internal fan is for air circulation to reduce vapor equilibrium time and to control the boundary layer conductance of the mirror surface (Campbell and Lewis, 1998). Additionally, an infrared thermometer measures the sample surface temperature. Both the dew-point and sample temperatures are then used to determine the water activity. The range of a commercially available dew-point meter is 0.030 to 1.000 a_w, with a resolution of ±0.001 a_w and accuracy of ±0.003 a_w. Measurement time is typically less than 5 min. The performance of the instrument should be routinely verified as described in the Support Protocol.

BASIC PROTOCOL

USING A DEW-POINT INSTRUMENT

Dew-point measurement is a primary method based on fundamental thermodynamics principles and as such does not require calibration. However, the instrument performance needs to be verified using salt standards and distilled water before sampling (see Support Protocol). To obtain accurate and reproducible water activity results with a dew-point instrument, temperature, sensor cleanliness, and sample preparation must be considered. Equipment should be used and maintained in accordance with the manufacturer's instruction manual and with good laboratory practice. If there are any concerns, the manufacturer of the instrument should be consulted. Guidelines common to dew-point instruments for proper water activity determinations are described in this protocol. The manufacturer's instructions should be referred to for specifics.

The type (e.g., liquid, solid, powder, gel, syrup, emulsion, granule) and range of food samples (raw ingredients to final products) for water activity measurement are immense. The amount of sample required for measurement is typically 5 to 10 ml. A homogeneous and representative sample should be prepared and placed into the sample cup. For the majority of samples, no preparation is necessary; the sample is simply placed into the cup. Multicomponent (e.g., muffin with raisins or pizza) and coated samples (e.g., breaded foods or chocolate-covered bar) may have to be sliced, crushed, or ground in order to obtain a representative sample. If sample preparation is necessary, then a consistent technique must be used with each sample to ensure reproducible results.

Materials

Sample
Dew-point water activity instrument (e.g., AquaLab Series 3, Decagon Devices)
Disposable sample cups (and optional lids; available from the instrument manufacturer)

1. Place a dew-point water activity instrument on a level surface and in a relatively temperature-stable environment. Allow instrument to warm up for ≥15 min after turning on for optimal performance.

   Performance verification should be conducted before sampling at least once per day, at each shift, or if readings become unstable (see Support Protocol).

2. Prepare a sample (e.g., liquid, solid, powder, gel, syrup, emulsion, granule) that is homogeneous and representative of the entire product.

   For detailed instructions on samples requiring special handling, see Critical Parameters and Troubleshooting, discussion of sample preparation.

3. Place sample (5 to 10 ml) in a disposable sample cup, completely covering the bottom of the cup, if possible. Place sample into sample cup in a timely manner to minimize moisture loss or gain during handling. If sample is not to be tested immediately, place a sample cup lid on the cup to prevent moisture transfer with the room atmosphere.

   Do not fill the sample cup more than half full. Overfilled cups will contaminate the sensors in the sampling chamber.

4. Wipe the cup rim with a lint-free tissue (e.g., Kimwipe), making sure that the rim and outside of the sample cup are clean and free of any sample material.

5. Place sample cup into the instrument. Check top lip of the cup to make sure no sample is protruding above its rim.

   Remember, an overfilled sample cup will contaminate the chamber's sensors.

6. Seal the measurement chamber, being especially careful not to splash or spill the sample and contaminate the chamber.

7. Start water activity measurement.

   This will start the read cycle, in which the dew-point temperature is repeatedly measured until vapor equilibrium is reached. The dew-point instrument will signal the operator when vapor equilibrium is achieved, and will display the final water activity and sample temperature on its screen.

   Length of read times may vary depending on temperature differences between the chamber and the sample, and on the properties of the sample.

8. Remove sample from instrument after measuring water activity.

   Never leave samples inside the chamber overnight or for extended periods of time, as this can contribute to contamination of the chamber.

PERFORMANCE VERIFICATION FOR DEW-POINT WATER ACTIVITY INSTRUMENT

Performance verification should be conducted before sampling at least once per day, at each shift, or if readings become unstable. Performance of the instrument is verified using salt standards that have known water activities. Either saturated or unsaturated salt solutions are suitable for verifying instrument performance. The choice of using saturated or unsaturated salt solutions is dependent upon the water activity range of the samples, temperature variability, cost, and ease of use. Saturated salt slurries have specific water activity values depending upon the chosen salt and the temperature. Some salts are hazardous and may not be appropriate in a food-testing laboratory. On the other hand, unsaturated salt solutions can be easily made to any water activity value desired. Unsaturated salt solutions (sodium chloride and lithium chloride) are much less temperature dependent than saturated salt slurries, and are thus stable over a wide temperature range. Instrument manufacturers provide certified unsaturated salt solutions at various water activity levels in individual prepackaged vials.

Additional Materials *(also see Basic Protocol)*

Saturated or unsaturated salt standards (see recipes)

Set up instrument

1. Place a dew-point water activity instrument on a level surface and in a relatively temperature-stable environment. Allow instrument to warm up for ≥15 min after turning on for optimal performance.
2. Choose a salt standard with a water activity level that is slightly below the water activity of sample to be measured. Make sure that salt standard is at ambient temperature before loading it into the instrument.
3. Fill a sample cup half full (~7 ml) with selected salt standard. Place sample into sample cup in a timely manner to minimize moisture loss or gain during handling.

   Do not fill the sample cup more than half full. Overfilled cups will contaminate the sensors in the sampling chamber.
4. Wipe the cup rim with a lint-free tissue, making sure that the rim and outside of the sample cup are clean and free of any sample material.
5. Place sample cup into the instrument. Check top lip of the cup to make sure no sample is protruding above its rim.
6. Seal the measurement chamber, being careful not to splash or spill the solution and contaminate the chamber.

Measure performance

7. Start the water activity measurement (see Basic Protocol, steps 7 and 8). Make two measurements.

   The water activity readings should be within ±0.003 a_w of the given value of the salt standard. If they are, proceed to step 10. If they are not, proceed to step 8.
8. If the readings are consistently outside the stated water activity values by more than ±0.003 a_w, the sensor chamber is contaminated. Clean chamber according to manufacturer's instructions.
9. Repeat step 7. If after cleaning, the readings are still outside of the stated water activity value by more than ±0.003 a_w, a calibration offset has probably occurred. Adjust the reading of a salt solution to its correct value according to manufacturer's instructions, then repeat step 7 again.

10. Prepare a sample cup half full of distilled water and make two readings.

    The second water activity reading should be 1.000 ± 0.003 a_w. If it is, the instrument is ready for use (see Basic Protocol). If it is not, proceed to step 11.

11. If the salt reading is correct and distilled water is not, it is probably due to slight contamination of the sensor chamber. Clean chamber according to manufacturer's instructions, then repeat step 7.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Saturated salt standards

Start with a salt and add water in small increments, stirring well with a spatula after each addition. Continue until the salt can absorb no more water, as evidenced by free liquid (where it will take on the shape of the container but will not easily pour). Store indefinitely at room temperature in a manner to prevent substantial moisture gain or evaporation losses.

When a salt standard is prepared so that it consists mostly of liquid with a few crystals in the bottom, it can result in a layer of less-than-saturated solution at the surface, which will produce a higher reading than anticipated. Conversely, solid crystals protruding above the surface of the liquid can lower the readings.

Table A2.2.1 lists the water activity of various salt slurries at 20° and 25°C (Greenspan, 1977). Greenspan provides additional information on these and other salts over a wider temperature range.

Saturated salt solutions in the form of salt slurries can be prepared by several other methods (see Stoloff, 1978; Mulvaney, 1995).

Unsaturated salt standards

Prepare salt solution at desired concentration (see Table A2.2.2 for NaCl or LiCl). Store indefinitely at room temperature in a manner to prevent substantial moisture gain or evaporation losses.

Unsaturated salt solutions of different concentrations make excellent standards. These solutions are easily made and are relatively unaffected by temperature over a wide range of concentrations (Chirife and Resnik, 1984). Table A2.2.2 lists the water activity of various sodium chloride and lithium chloride solutions according to Robinson and Stokes (1965). The data in Robinson and Stokes can be used to compute the a_w for other unsaturated salt solutions.

Dew-point instrument manufacturers also provide certified unsaturated salt solutions at various water activity levels in individual prepackaged vials.

Table A2.2.1 Water Activity of Selected Saturated Salt Standards[a]

Salt	a_w at 20°C	a_w at 25°C
LiCl	0.113 ± 0.003	0.113 ± 0.003
MgCl	0.331 ± 0.002	0.328 ± 0.002
K_2CO_3	0.432 ± 0.003	0.432 ± 0.004
$Mg(NO_3)_2$	0.544 ± 0.002	0.529 ± 0.002
NaCl	0.755 ± 0.001	0.753 ± 0.001
KCl	0.851 ± 0.003	0.843 ± 0.003
K_2SO_4	0.976 ± 0.005	0.973 ± 0.005

[a]Adapted from Greenspan (1977). Numbers rounded to nearest thousandth.

Table A2.2.2 Water Activity of Unsaturated Sodium Chloride and Lithium Chloride Solutions at 25°C[a]

Sodium chloride		Lithium chloride	
Molality	a_w	Molality	a_w
0.1	0.996	0.1	0.997
0.2	0.993	0.5	0.983
0.3	0.990	1.0	0.964
0.4	0.986	1.4	0.948
0.5	0.983	2.0	0.921
0.6	0.980	2.5	0.897
0.7	0.976	3.0	0.870
0.8	0.973	3.5	0.842
0.9	0.970	4.0	0.812
1.0	0.966	4.5	0.780
1.2	0.960	5.0	0.747
1.4	0.953	5.5	0.713
1.6	0.946	6.0	0.679
1.8	0.938	7.0	0.609
2.0	0.931	8.0	0.539
2.2	0.924	9.0	0.473
2.4	0.916	10.0	0.412
2.6	0.908	11.0	0.356
2.8	0.901	12.0	0.307
3.0	0.893	13.0	0.266
3.2	0.885	14.0	0.230
3.4	0.876	15.0	0.200
3.6	0.868	16.0	0.175
3.8	0.860	17.0	0.155
4.0	0.851	18.0	0.138
5.0	0.807	19.0	0.123
6.0	0.760	20.0	0.110

[a]Adapted from Robinson and Stokes (1965).

COMMENTARY

Background Information

Water activity (a_w) is an important property for food safety. It predicts food safety and stability with respect to microbial growth, chemical or biochemical reaction rates, and physical properties (Figure A2.2.1). By measuring the water activity of foodstuffs, it is possible to predict which microorganisms will be potential sources of spoilage and infection. Controlling water activity is also an important way to maintain the chemical stability of foods. Water may influence chemical reactivity in different ways. It may act as a solvent or reactant, or change the mobility of the reactants by affecting the viscosity of the food system (Leung, 1987). Water activity influences nonenzymatic browning, lipid oxidation, degradation of vitamins, enzymatic reactions, protein denaturation, starch gelatinization, and starch retrogradation. Finally, a_w plays a significant role in the physical properties of foods, such as texture and shelf life. Water activity is the driving force for moisture migration within a food sample.

Reliable laboratory instrumentation is required to guarantee the safety of food products and enforce government regulations. Two main types of commercial instruments are available. One uses chilled mirror dew-point technology, while the other measures relative humidity with sensors that detect changes in electrical resistance or capacitance. Each has advantages and disadvantages. The methods vary in accuracy, repeatability, speed of measurement, stability in calibration, linearity, and convenience of use.

Water activity is derived from fundamental principles of thermodynamics and physical chemistry. It is defined from the equilibrium state:

$\mu = \mu_0 + (RT \times \ln[f/f_o])$

where μ is the chemical potential of the system, i.e., thermodynamic activity or energy per mole of substance; μ_0 is the chemical potential of the pure material at the temperature T; R is the gas constant; f is the fugacity or the escaping tendency of a substance; and f_0 is the escaping tendency of pure material (van den Berg and Bruin, 1981). The activity of a species is defined as $a = f/f_0$. When dealing with water, a subscript is designated for the substance, $a_w = f/f_0$, where a_w is activity of water, or the escaping tendency of water in a system divided by the escaping tendency of pure substance with no radius of curvature. For practical purposes, under most conditions in which foods are found, the fugacity is closely approximated by the vapor pressure ($f \approx p$):

$a_w = f/f_0 \cong p/p_0$

Equilibrium is obtained in a system when μ is the same everywhere in the system. Equilibrium between the liquid and the vapor phases implies that μ is the same in both phases. It is this fact that allows the measurement of the vapor phase to determine the water activity of the sample.

The water activity of a food describes the energy status of water in a food and, hence, its availability to act as a solvent and participate in chemical or biochemical reactions (Labuza, 1977). Figure A2.2.1 is a global stability map of foods, showing stability as a function of a_w (Labuza, 1970). Water's ability to act as a solvent, medium, and reactant increases with increasing water activity (Labuza, 1975).

Critical Parameters and Troubleshooting

To obtain accurate and reproducible water activity results with a dew-point instrument, the temperature, sensor cleanliness, and sample preparation must be considered. Temperature influences water activity measurements in two ways; one is its effect on the water activity in the sample and the second is the effect on the dew-point measurement.

Temperature effect on sample water activity

Water activity is temperature dependent. Temperature changes water activity due to changes in water binding, dissociation of water, solubility of solutes in water, or the state of the matrix. Although solubility of solutes can be a controlling factor, control is usually from the state of the matrix. Since the state of the matrix (glassy versus rubbery) is dependent on temperature, one should not be surprised that temperature affects the water activity of the food. The effect of temperature on the water activity of a food is product specific. Some products increase a_w with temperature, others decrease a_w with increasing temperature, while most high-moisture foods have negligible change with temperature. Therefore, one cannot predict even the direction of the change of water activity with temperature, since it depends on how temperature affects the factors that control water activity in the food.

The importance of temperature control is significant for laboratory comparisons, accelerated shelf-life studies, and packaging requirements. Also, temperature may be essential when measuring a_w levels near critical values, especially for government regulations or critical control points. The precision required in water activity applications will determine the need for temperature control. Several reasons for sample temperature control are:

Research purposes. These include studies that examine the effects of temperature on the a_w of a sample or follow accelerated shelf life conditions, as well as those that compare the a_w of different samples independent of temperature. There are many shelf life, packaging, and isotherm studies in which the added feature of temperature control would be very beneficial.

To comply with government or internal regulations for specific products. Though the a_w of most products varies by less than ± 0.002 a_w per °C, some regulations require measurement at a specific temperature. The most common specification is 25°C, though 20°C is sometimes indicated.

To minimize extreme ambient temperature fluctuations. If the laboratory and dew-point instrument temperatures fluctuate by as much as ±5°C daily, water activity readings may vary by $\pm 0.01 a_w$. Often, this much uncertainty in sample a_w is unacceptable, so there is a need for a temperature-controlled model.

Chilled mirror dew-point instruments are available in a variety of temperature-controlled models from the manufacturer.

Temperature effect on dew-point measurement

Temperature must be defined for water activity measurements. Changes in sample temperature will cause the water activity readings

to change until the sample temperature stabilizes. When the sample temperature stabilizes within one or two degrees of the chamber temperature, then accurate and rapid measurements are made. Samples that are 4°C colder or warmer than the instrument will need to equilibrate to the instrument temperature before accurate readings can be made. If a sample is frozen or refrigerated before testing, it needs to be brought back to ambient temperature so that condensation moisture can be reabsorbed into the product. Samples with high water activity that are above the chamber temperature can cause condensation within the chamber, which will adversely affect subsequent readings. Some instruments display a warning message if the sample temperature is above the chamber temperature.

Sensor performance

The accuracy of a dew-point instrument is vitally dependent on keeping the instrument clean. Commercial dew-point instruments will provide accurate water activity measurements as long as their internal sensors are not contaminated by improperly prepared samples. If the sensors are contaminated, they must be cleaned. Careful preparation and loading of samples will lengthen the time between cleanings. The rim and outside of the sample cup should be kept clean. Any excess sample material should be wiped from the rim of the cup with a clean lint-free tissue. The rim of the cup must be able to form a vapor seal with the sensor block when the water activity measurement is started. Any sample material left on the cup rim will be transferred to the chamber block, preventing this seal and contaminating future samples. The sample cup should not be filled more than half full, as overfilled cups are likely to result in spilled sample and contamination of the sensors in the chamber. When measuring liquid samples, it is important to be gentle when inserting the sample so as not to spill the solution out of the dish. If the drawer is pushed or pulled too quickly, the solution will spill and contaminate the chamber. Sensor performance and cleanliness is verified by using salt standards and distilled water (see Support Protocol).

Sample preparation

For most foods, water activity is an important property of stability and safety. Thus, samples ranging from raw ingredients to finished goods should be analyzed for water activity. The sample to be tested must be representative of the entire product. Most samples do not need any further preparation; they can simply be placed in the cup and measured for water activity. Some precautions should be taken for multicomponent and coated samples to ensure that a representative and homogeneous sample is tested.

For multicomponent and coated samples there are two methods of preparation, depending on the purpose of the water activity analysis. The first method is to obtain a representative sample of the entire product by crushing or grinding the sample. If the sample is to be crushed or ground, then a consistent technique is necessary to ensure reproducible results. For example, if one sample is ground for 15 sec and a second sample is ground for 1 min, then the second sample will have smaller particles with more surface area and a greater chance of gaining or losing moisture to the environment. In addition, the time between grinding and the water activity measurement should be consistent, or additional precautions should be taken to prevent moisture exchange with the room air.

The second method is to separate the components and measure each individually. Since water activity is a driving force for moisture migration, it might be necessary to know the water activity values for the individual components. For example, in a flake cereal with fruit pieces, it might be important to know the individual component water activities. The water activity of the cereal flakes and fruit pieces should be as close as possible to prevent moisture migration, which would cause the flakes to become soggy and the fruit pieces to become hard and brittle.

In addition to equilibrium between the liquid-phase water in the sample and the vapor phase, the internal moisture equilibrium of the sample is important. If a system is not at internal moisture equilibrium, one might measure a steady vapor pressure (over the period of measurement) that is not the true water activity of the system. An example of this might be a baked good or a multicomponent food. Initially out of the oven, a baked good is not at internal equilibrium; the outer surface is at a lower water activity than the center of the baked good. One must wait a period of time in order for the water to migrate and the system to come to internal equilibrium. It is therefore important to remember the restriction of the definition of water activity to equilibrium.

Limitations

Chilled mirror dew-point instruments have limitations with certain volatile compounds

that co-condense on the mirror surface with water vapor. Samples with high levels of propylene glycol require special sampling procedures to obtain accurate readings. Samples that contain propylene glycol in concentrations >10% will not damage the instrument, but a_w values for consecutive samples will not be accurate. Propylene glycol condenses on the mirror during the reading process, but does not evaporate from the mirror as water does. As a result, the very first reading will be somewhat accurate, but subsequent readings will not be accurate unless propylene glycol is cleared from the chamber after each measurement. Running a sample consisting of activated charcoal after each sample containing propylene glycol accomplishes this. Dew-point instruments will give accurate readings on most samples containing alcohols, except those containing ethanol. Samples containing ethanol will yield elevated water activity results.

Anticipated Results

Commercial water activity instruments that use the chilled mirror dew-point method described above measure water activity values between 0.030 and 1.000 a_w. They attain a precision of 0.001 a_w with an accuracy of ±0.003 a_w and a resolution of ±0.001 a_w.

Time Considerations

Measuring both the dew point and sample temperature eliminates the need for complete thermal equilibrium and reduces measurement times to <5 min for most samples. Because water activity is an equilibrium measurement, multicomponent samples, samples that have outside coatings, or high-fat samples may take longer to equilibrate. In addition, some extremely dry samples, dehydrated samples, highly viscous samples, or glassy compositions may have increased read times due to their moisture sorption characteristics. Samples like these may take >5 min to give an accurate reading or may require multiple readings. To reduce the time needed to take an a_w reading for coated, dried, or glassy samples, one can crush, slice, or grind the sample before placing it in the sample cup. This increases the surface area of the sample, thus increasing the equilibration rate. When samples are crushed, ground, or sliced, the method should be consistent in order to ensure reproducible results.

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Internet Resources

www.decagon.com/aqualab/

Web site containing dew-point method and theory information.

Contributed by Anthony J. Fontana
Decagon Devices
Pullman, Washington

Measurement of Water Activity Using Isopiestic Method

Water is an important constituent of all foods. In the middle of the twentieth century, scientists began to discover the existence of a relationship between the water contained in a food and its relative tendency to spoil. They also began to realize that the chemical potential of water is related to its vapor pressure relative to that of pure water (i.e., the relative vapor pressure; RVP). This RVP was termed "water activity" or a_w. Scott (1957) clearly stated that the a_w of a medium correlated with the deterioration of food stability due to the growth of microorganisms. Thus, it is possible to develop generalized rules or limits for the stability of foods using a_w. Food scientists, therefore, started to emphasize a_w along with water content. Since then, the scientific community has explored the great significance of a_w in determining the physical characteristics, processes, shelf life, and sensory properties of foods. Recently, Rahman and Labuza (1999) have presented a detailed review on this aspect of a_w. Details of the various measurement techniques are presented by Labuza et al. (1976), Rizvi (1995), Rahman (1995), and Bell and Labuza (2000).

Water activity, a thermodynamic property, is defined as the ratio of the vapor pressure of water in a system to the vapor pressure of pure water at equal temperature, or the equilibrium relative humidity of the air surrounding the system at equal temperature. Thus, a_w can be expressed as follows:

$$a_w = \frac{\left(P_w^v\right)_{sy}}{\left(P^v\right)_w} = \text{ERH}$$

Equation A2.3.1

where a_w is water activity ($0.0 \le a_w \le 1.0$), $(P_w^v)_{sy}$ is the vapor pressure of water in the system (Rahman, 1995), $(P^v)_w$ is the vapor pressure of pure water, and ERH is equilibrium relative humidity of air, all of which are specific for a given temperature.

Isopiestic determination is one of the most commonly used methods for measuring food a_w. In this method a sample of known mass is stored in a closed chamber and allowed to reach equilibrium with an atmosphere of known ERH (or equilibrate with a standard of known a_w). In the first protocol (see Basic Protocol), a standard salt solution, for which a_w is well established, is used to control this atmosphere. The a_w of the sample is then determined by equilibration with the resulting atmosphere. In the second protocol (see Alternate Protocol), the isopiestic determination of a_w is accomplished by equilibration of the sample with a reference material, for which the relationship between water content and a_w is known. The condition of equilibrium is determined by reweighing the sample at intervals until constant mass is reached. The moisture content of the sample is then determined either directly or by calculation from the reference material's original moisture content and change in mass. Unsaturated salt solutions of known ERH can also be used to equilibrate the samples; however, this requires estimation of the ERH of the jars at the end of the equilibration by measuring the exact concentration of the salt solution, which may be tedious.

In the isopiestic method, often the purpose is to characterize how the a_w of a sample depends upon its gravimetric water content. This curve, which relates a_w and water content, is known as the "sorption isotherm." Knowledge of the sorption isotherm allows estimation of a_w if a samples' moisture content is known. This is useful, as it is often easier and more rapid to determine moisture content than a_w (Rahman, 1995).

Contributed by Shyam S. Sablani, M. Shatiur Rahman, and Theodore P. Labuza

Current Protocols in Food Analytical Chemistry (2001) A2.3.1-A2.3.10

BASIC PROTOCOL

ISOPIESTIC DETERMINATION OF WATER ACTIVITY (a_w) BY EQUILIBRATION WITH SATURATED SALTS

In this method of measuring sample a_w, a preweighed sample is placed in a closed chamber and maintained at constant temperature at a known a_w (ERH) controlled by a saturated salt solution. Equilibrium is established between the environment and the sample, as determined by reweighing the sample at intervals until constant mass is established. The moisture content of the sample is then determined by drying (*UNIT A1.1*) or Karl Fischer titration (*UNIT A1.2*). Measurements are made with different saturated salt solutions, and a graph is prepared of water content versus a_w. Microcrystalline cellulose (Avicel; FMC) can be selected for the reference material to evaluate the precision of the method. Advantages of MCC as a reference material are identified by Spiess and Wolf (1987). It is stable in its crystalline structure in a temperature range of −18° to 80°C, with minimal changes in its sorption characteristics; however, when exposed to temperatures above 100°C, the sorption properties may change and thermal degradation is expected to begin at temperatures above 120°C. MCC is stable in its sorption properties after two to three repeated adsorption and desorption cycles. Other details can be found in Bell and Labuza (2000).

Materials

Saturated salt solution (Table A2.3.1 and Table A2.3.2)
1 to 2 g sample
Tolulene, sodium azide, or thymol in a test tube or small bottle (optional)

Closed chamber (e.g., desiccator, tropical fish tank with lid, glass jars; Fig. A2.3.1)
Mechanism for maintaining temperature (e.g., thermostatted cabinet, room, or water bath)
Petri dish or weighing bottle
Glass jar or support plate

Additional reagents and equipment for gravimetric determination of water content by drying (*UNIT A1.1*) or Karl Fischer titration (*UNIT A1.2*)

Prepare salt solutions and chamber

1. Prepare different saturated salt solutions with distilled water in jars (to create different relative humidities) at a temperature (~10°C) higher than that used for equilibration. Place the jar in a closed chamber (e.g., dessicator, tropical fish tank with lid; Fig. A2.3.1).

Table A2.3.1 Water Activities (a_w) of Selected Saturated Salt Solutions at Various Temperatures[a,b]

Salts	5°C	10°C	20°C	25°C	30°C	40°C	50°C
Ammonium sulfate ($[NH_2]_2SO_4$)	0.824	0.821	0.813	0.810	0.806	0.799	0.792
Lithium chloride (LiCl)	0.113	0.113	0.113	0.113	0.113	0.112	0.111
Magnesium chloride ($MgCl_2$)	0.336	0.335	0.331	0.328	0.324	0.316	0.305
Magnesium nitrate ($Mg[NO_3]_2$)	0.589	0.574	0.544	0.529	0.514	0.484	0.454
Potassium acetate ($KC_2H_3O_2$)	—	0.234	0.231	0.225	0.216	—	—
Potassium carbonate (K_2CO_3)	0.431	0.431	0.432	0.432	0.432	—	—
Potassium chloride (KCl)	0.877	0.868	0.851	0.843	0.836	0.823	0.812
Potassium iodide (KI)	0.733	0.721	0.699	0.689	0.679	0.661	0.645
Potassium nitrate (KNO_3)	0.963	0.960	0.946	0.936	0.923	0.891	0.848
Potassium sulfate (K_2SO_4)	0.985	0.982	0.976	0.973	0.970	0.964	0.958
Sodium chloride (NaCl)	0.757	0.757	0.755	0.753	0.751	0.747	0.744

[a]Source is Greenspan (1971).
[b]Dashes indicate that data is not available.

Maintain a layer of solid salts in the slurries during the whole period of equilibration to confirm that the solutions always remain saturated. Generally, the solutions should be slushes with ~4 mm of solution over the crystal layer. At each equilibration temperature used, the a_w (ERH) of the atmosphere above the saturated solution will be different (Table A2.3.1 and Table A2.3.2).

Solutions are made at a higher temperature than equilibration to insure that they are saturated slurries when cooled (see Critical Parameters).

Table A2.3.2 Regression Equations for a_W of Selected Saturated Salt Solutions at Different Temperatures[a]

Salts	Regression equation[a]	r^2
Lithium chloride (LiCl)	$\ln (a_w) = (500.95 \times 1/T) - 3.85$	0.976
Magnesium chloride ($MgCl_2$)	$\ln (a_w) = (303.35 \times 1/T) - 2.13$	0.995
Magnesium nitrate ($MgNO_3$)	$\ln (a_w) = (356.60 \times 1/T) - 1.82$	0.987
Potassium acetate ($KC_2H_3O_2$)	$\ln (a_w) = (861.39 \times 1/T) - 4.33$	0.965
Potassium carbonate (K_2CO_3)	$\ln (a_w) = (145.00 \times 1/T) - 1.30$	0.967
Potassium chloride (KCl)	$\ln (a_w) = (367.58 \times 1/T) - 1.39$	0.967
Sodium chloride (NaCl)	$\ln (a_w) = (228.92 \times 1/T) - 1.04$	0.961
Sodium nitrate ($NaNO_2$)	$\ln (a_w) = (435.96 \times 1/T) - 1.88$	0.974

[a]Source: Labuza et al. (1985).
[b]Temperature (T) is in degrees Kelvin.

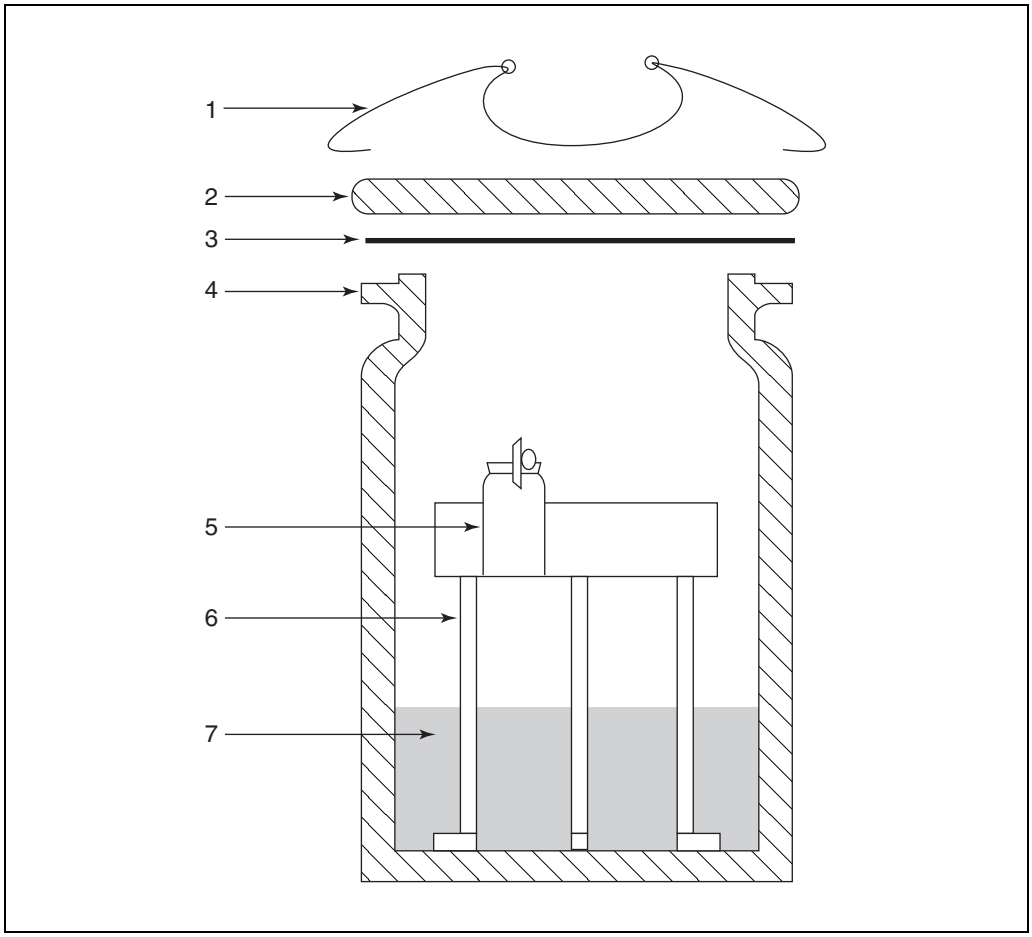

Figure A2.3.1 Standardized sorption apparatus: (1) locking clamp; (2) glass lid; (3) rubber seal ring; (4) sorption container, glass; (5) weighing bottle with ground-in stopper; (6) petri dish on trivet; (7) saturated salt solution. Adapted from Spiess and Wolf (1987) courtesy of Marcel Dekker.

2. Equilibrate the closed chamber to a constant temperature (e.g., by storing it in a thermostatted cabinet, room, or water bath).

   The saturation of the solution is temperature dependent; therefore, a_w (the parameter being controlled) is temperature dependent. Thus, temperature must be both precisely known and constant.

3. Weigh the sample (1 to 2 g) in a Petri dish or weighing bottle and place in the jar and transfer the jar to the chamber.

   In some cases, one or a number of weighing bottles are placed on a petri dish and then the petri dish is placed in the jar (Fig. A2.3.1).

   The sample can also be hung (Fig. A2.3.2), to facilitate weighing without removal.

4. (Optional) Prevent microbial growth by placing a test tube or small bottle containing toluene, sodium azide, phenyl acetate, or thymol (for non-fatty products) inside the closed chamber to prevent mold growth on the sample at a_w>0.75.

   CAUTION: *Sodium azide (NaN_3) and phenyl acetate are poisonous. Toluene is not recommended since it is carcinogenic. Take appropriate precautions when using any of these chemicals. See APPENDIX 2B for more information.*

5. Seal the jars and close the chamber/cabinet.

   Rubber seal rings are used in the case of jars, while silicon grease is used in the case of desiccators. Be sure that the sealing is tight enough to prevent leakage of gas from the chamber. It may be difficult to prevent leakage or damage of the glass container at high temperature; instead, metal cells with lids and gaskets can be used.

   Several jars can be placed in the same cabinet. Equilibration at different temperatures requires separate samples and cabinets.

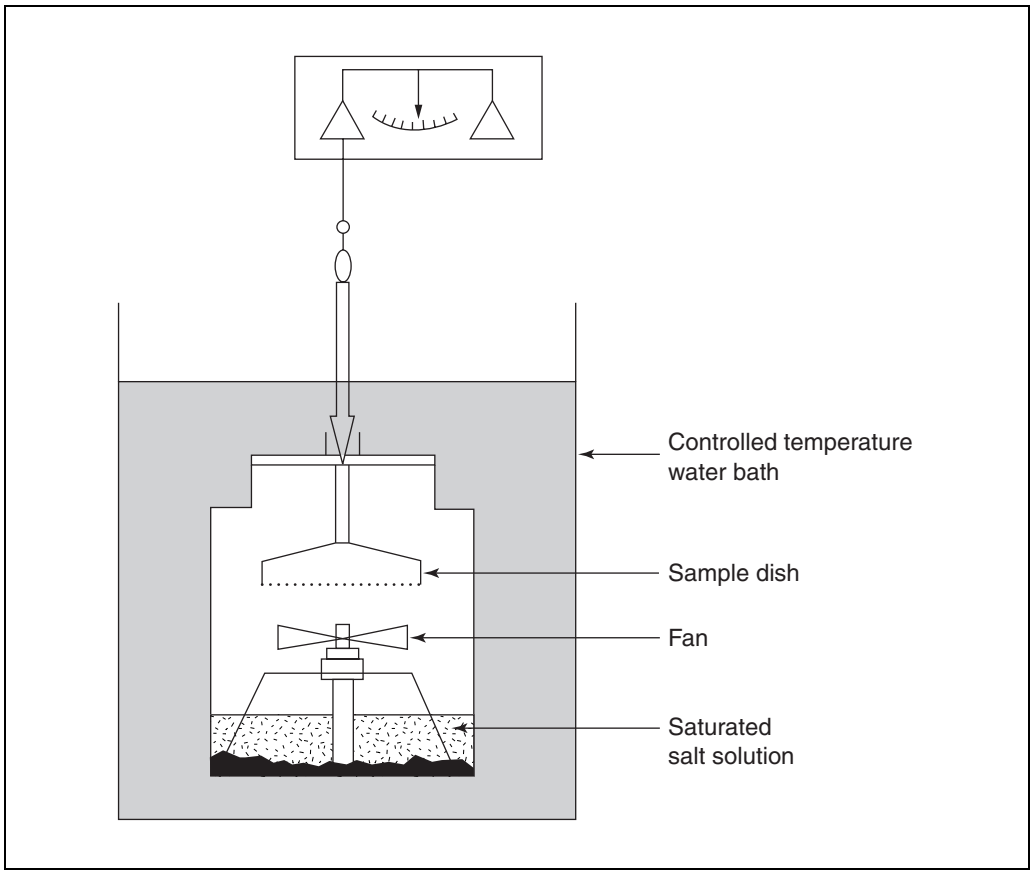

Figure A2.3.2 An apparatus with circulated air chamber and in situ weighting system for measurement of water activity (a_w). Adapted from with permission Igbeka and Blaisdell (1982).

Establish equilibrium

6. Establish that equilibrium has occurred by weighing the sample at intervals until there is no change in mass.

   Samples are weighed at 2 to 3 day intervals until equilibration. Usually this takes ~10 days at a_w< 0.75 or ~21 days at a_w>0.75.

7. Measure moisture content of the equilibrated sample by weighing before and after drying either in an air or vacuum oven as described in *UNIT A1.1* or by Karl Fischer titration (*UNIT A1.2*).

   Air drying at 105°C or vacuum drying at 70°C for more than 18 hr is usually used to determine the solids mass in the sample (UNIT A1.1); however, Karl Fischer titration is recommended, due to possible temperature abuse in the oven method (refer to UNIT A1.2).

   The infrared moisture analyser may also be used for quick determination of water content although it gives a value that is different than the other two methods (subject of a future unit on NIR). This may be useful if a rapid IR method is used in a production line so one can correlate line moisture with true moisture.

Determine a_w

8. Determine the a_w of the sample, as found in Table A2.3.1 and Table A2.3.2.

   The a_w of the sample is equal to the a_w of the saturated salt solution at equilibrium.

9. Determine the moisture content of equilibrated food samples from several jars with different saturated salt solutions (and therefore different a_w).

   Approximately 8 determinations are required to generate a valid sorption isotherm.

   The experiments are performed at different temperatures by repeating the above procedure (steps 1 to 8) or multiple chambers are set at different temperatures in parallel (usually at least three temperatures ranging from 10° to 60°C).

10. Plot sorption isotherms relating a_w versus moisture content.

    The BET (Eq. A2.3.4) or GAB (Eq. A2.3.5) equations relating water content of the sample and a_w can be used to model isotherms. The constants in BET equations have to be determined for each temperature while GAB equation can accommodate the temperature effect as well (see Anticipated Results). Any commercial software (e.g., SAS, Statistica, S-plus, Minitab) with nonlinear regression feature can be used to determine constants in the BET or GAB equations.

ISOPIESTIC DETERMINATION WATER ACTIVITY BY EQUILIBRATION WITH STANDARDS OF KNOWN WATER ACTIVITY (a_w)

ALTERNATE PROTOCOL

This method involves preparation of a standard curve by equilibration of a specific amount of dry standard material in duplicate or triplicate over different saturated salt solutions. The standard curve is a plot of a_w versus water content of the standard material. The standard must be stable during reuse of the material, and the chamber used should be exactly the same as will be used later. For the measurement, the size of each standard should be in a controlled narrow range, (e.g., ~1.6 ± 0.1 g). Once the standard curve is made, dry aliquots of the same mass of standard are equilibrated over a large quantity of the food material (~10 to 20 g). The moisture content of the standard material is then measured (e.g., by mass gain) and a_w is estimated from the standard curve (Vos and Labuza, 1974). This method avoids preparation or storing of saturated salts for each determination; moreover, use of a standard shortens the equilibration, thus requiring less time for measurement, and less abuse of temperature for sample as well as standard.

This method uses the same materials and apparatus as described above (see Basic Protocol 1). In addition a standard material, such as microcrystalline cellulose (MCC), starch, filter paper, glycerol, or polyethylene glycol is needed for this protocol. The standard material is selected based on the commercial availability of a material with high purity and consistency over time, moreover, the standard must be stable during reuse of the material. Thus, MCC is recommended (Spiess and Wolf, 1987). Once the standard is chosen, it is used as a replacement for the sample in the procedure described above (see Basic Protocol 1), while the sample replaces the salt.

Additional Materials *(also see Basic Protocol)*

Adsorbent: phosphorus pentoxide (P_2O_5) or calcium chloride ($CaCl_2$)
~1.6 ± 0.1 g standard material (e.g., MCC, starch, filter paper, glycerol, polyethylene glycol)

1. Dry the weighing bottles together with ~1.6 ± 0.1 g standard in a vacuum oven at 70°C for 18 hr using phosphorus pentoxide (P_2O_5) or calcium chloride ($CaCl_2$) as adsorbent. Record the mass.

   Since the a_w of saturated salt solutions is temperature dependent, it is important to maintain a constant temperature during experiments.

2. Equilibrate the standard at known temperature with a range of saturated salt solutions to establish different relative humidities as described (see Basic Protocol).

   Samples are weighed at 20 to 3 day intervals until equilibration. Usually this takes ~10 days at a_w< 0.75 or ~21 days at a_w>0.75.

3. After equilibration reweigh the standard and determine moisture content using any drying methods (*UNIT A1.1*). Plot water content values of the standard against a_w values of the saturated salt solutions.

   The nonlinear standard curve generated can be modeled using the GAB equation (Bell and Labuza, 2000).

4. Place a large excess of the food sample (at least 10 g food/1 g standard) into a closed chamber as the source of water. Also place dry standard of the same mass as that weighed in step 1.

   The relative amounts of sample and standard material have to be different because the moisture transferred from the food sample to the standard (characterized by the mass gain of the standard) must not result in a significant change in the vapor pressure maintained by the sample. Essentially, the sample replaces the salt solution in the Basic Protocol to create constant a_w in the closed chamber.

   The standard is weighed to an exact amount similar to the amount used in the isotherm determination (step 1). The standard is placed into the closed chamber which contains the unknown sample as the a_w source and is equilibrated at a constant temperature. The key point is that equilibrium does not take as much time as the procedure using equilibration with saturated salt solutions (see Basic Protocol); i.e., the mass change can be determined in 24 hr if the calibration curve is also done with the same mass of standard and equilibrated for the same amount of time (step 3).

5. Determine the a_w of the food sample, under the conditions of measurement, by estimating from the standard curve (step 3) based on the change in moisture content.

   The a_w of the food sample is equal to the a_w of the standard, assuming equilibrium (or near equilibrium) is achieved.

COMMENTARY

Background Information

Water sorption isotherm

The water sorption isotherm shows the dependence of water content on the a_w of a given sample at a specified temperature. It is usually presented in graphical form or as an equation. The difference in the equilibrium water content between the adsorption and desorption curves is called hysteresis and is shown in Figure A2.3.3. In foods, a variety of hysteresis loop shapes can be observed, depending on the type of food and the temperature. The principal factors affecting hysteresis are composition of the product, isotherm temperature, storage time before isotherm measurement, pretreatments, drying temperature, and the number of successive adsorption and desorption cycles. It should be noted that the occurrence of hysteresis indicates that either the adsorption or desorption curve is not at true equilibrium or that some material in the product changed state in the process (e.g., the crystallization of sugars during adsorption).

Water activity shift and break in isotherm

Typical a_w shifts with temperature are shown in Figure A2.3.4. The a_w shift by temperature is mainly due to the change in the excess enthalpy of water binding, dissociation of water, physical state of water, or increase in solubility of solute in water as temperature increases. It is widely accepted that an increase in temperature results in a decreased equilibrium moisture content in foods. In some products, at a_w>0.5, there is an inversion of the effect of temperature (i.e., equilibrium moisture content increased with temperature) mainly due to an increase in solubility of sugars in water.

The isotherm shift due to temperature at a constant moisture content can usually be estimated by the well-known Clausius-Clapeyron-equation as:

$$\ln\frac{(a_w)_2}{(a_w)_1} = \frac{q+\lambda_w}{R}\left[\frac{1}{T_2}-\frac{1}{T_1}\right]$$

Equation A2.3.2

The slope of a plot of $\ln(a_w)$ versus $1/T$ should give:

$$\text{slope} = \left[\frac{q=\lambda_w}{R}\right]$$

Equation A2.3.3

where T is the temperature in Kelvin, q is the excess heat of sorption in kilojoules per kilogram, λ_w is the latent heat of vaporization for water in kilojoules per kilogram, and R is the ideal gas constant (0.4619 kJ kg^{-1} K^{-1}). In a

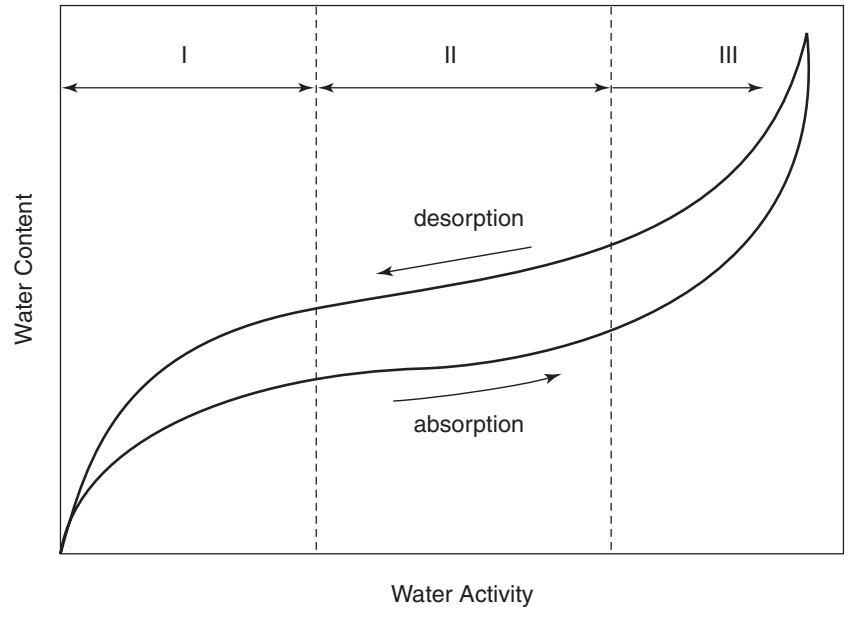

Figure A2.3.3 Sorption isotherm for typical food product showing hysteresis. Reprinted from Rahman and Labuza (1999), courtesy of Marcel Dekker.

pure component isotherm, the change of solute from the amorphous to a crystal state affects the isotherm. A break is also observed in the isotherm. In some foods one part of the solute (salt and sugar) is bound to a polymer (protein and starch) and the other part is crystalline or amorphous. The change of solute from an amorphous to a crystalline state or loss of solute bound to polymer also causes a break in the isotherm.

The main disadvantages of the methods discussed above are the slowness of the equilibrium (due to lower diffusion and the loss of conditioned atmosphere each time the chamber is opened to remove the sample for weighing) and the potential for this delay to lead to mold or bacterial growth on the samples and consequent invalidation of the results (Rahman, 1995).

Improvement to enhance the equilibrium time can be achieved by creating a vacuum in the desiccator (as was done by Fett, 1973); however, one has to be careful not to cause the salt solution to spatter during this process or to blow the sample out of the weighing dish when the vacuum is released. Other methods include circulating the atmosphere with a fan or pump (again caution is needed to prevent entrainment of salts into the air, which can deposit on the sample), or increasing the surface area of the sample by slicing or breaking it into pieces and spreading in a thin layer over a large surface area. The loss of a controlled ERH atmosphere during weighing can be avoided by weighing the sample in situ by a scale attached to the sample holder. Such an apparatus (Fig. A2.3.2) consists of a magnetic stirrer unit, equilibrium chamber in a constant temperature bath, and a sensitive weighing balance; however, initial evacuation or vacuum is the most effective method for enhancing equilibration. Systems are commercially available for the fast measurements by the isopiestic method. The Sorbostat and Sorbostate de Luxe from Chemcotec AG consists of a small desiccator in which a perforated aluminum disk carrying up to 8 to 16 weighing bottles is inserted together with a small magnetically operated ventilator. The electrolyte solution is accommodated in the bottom of the desiccator and can be stirred by means of a magnetic stirrer driven by a low-voltage synchronous type motor. The main features of this device are the magnetically operated opening and closing mechanism for the weighing bottles inside the closed desiccator and the ability to handle many samples concurrently. An integrated microbalance system by VTI Corporation, Autosorp by Biosystems, and Dynamic Vapour Sorption (DVS) by Surface Measurement Systems are also commercially available for fast measurements. The main features of these systems are insulated chambers with controlled-humidity air circulation over the sample, and a built-in electronic precision balance. All the systems commercially available have the computer software facilities for data analysis and the interpretation of results.

Critical Parameters

The salt solutions should be prepared with hot distilled water, continually adding more salt with stirring until no more dissolves. Excess should then be added to make the solution a slurry. The final solution is cooled and allowed

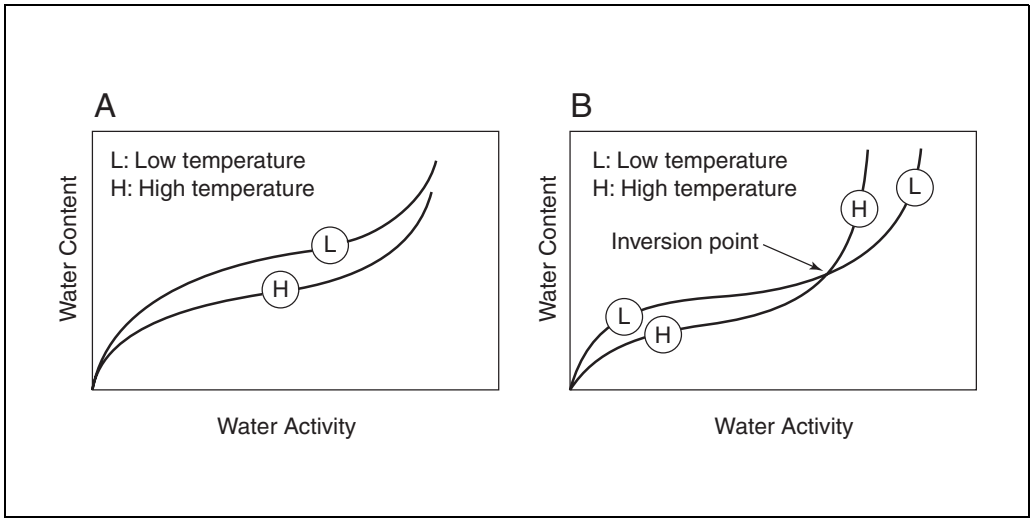

Figure A2.3.4 Effect of temperature on water sorption isotherm. (**A**) Shift without intersection. (**B**) Shift showing the point of intersection. Reprinted from Rahman and Labuza (1999), courtesy of Marcel Dekker.

to stand a few days at the desired temperature in closed conditions. The slurry should be stirred briefly once a day unless this disturbs equilibrium too much. It is important to keep the solution as a slurry during the entire period of equilibration. Crystals of salts may be present at the beginning of experiment, but may be dissolved when water from the sample is condensed in the solution. This can be overcome by adding salts in the solution when the chamber is opened for weighing the sample or better by using a large excess of slurry to amount of sample. Since only the a_w of the saturated solution is known, it is important that undissolved crystals of the salts are present during and after equilibration, as this is the only clear indicator of saturation. The relative humidity of the solution after saturation does not change significantly.

Microbial growth at high a_w can be avoided by using sodium azide in a special support.

It is important to make sure there is no leakage of gas from the chamber.

Sample size is important for the time of equilibration. The sample size usually used is <5 g.

Anticipated Results

Factors affecting a_w are the types of components in food, the physicochemical state of components, porous structure, temperature, total pressure, and surface tension (Rahman, 1995; Rizvi, 1995; Bell and Labuza, 2000); however, the effect of surface tension is very slight. Brunauer et al. (1940) classified adsorption isotherms of materials into five general types (Fig. A2.3.5). If water-soluble crystalline components are present in foods (e.g., sugars or salts), the isotherm appears as concave shape type III. Most other foods containing higher molecular weight solute result in sigmoid isotherm type II. Type I is indicative of a nonswelling porous solid, such as silicate anticaking agents. Types II and III are closely related to types IV and V, except that maximum adsorption occurs at some pressure lower than the vapor pressure of sample.

For most practical purposes, the isotherm can be modeled by an empirical (Brunauer, Emmett and Teller; BET) or theoretical (Guggenheim, Anderson, and DeBoer; GAB) equation (see below); however, none of the isotherm models in the literature is valid over the entire a_w range of 0 to 1. The GAB model is one of the most widely accepted models for foods over a wide range of a_w (from 0.10 to 0.90). The details of the different isotherm models with their parameters have been compiled by Rahman (1995). The BET (Eq. A2.3.4) and GAB (Eq. A2.3.5) equations are given as follows:

$$M_w = \frac{M_m B a_w}{\left(1 - a\left[1 + \left(B - 1\right)a_w\right]\right)}$$

Equation A2.3.4

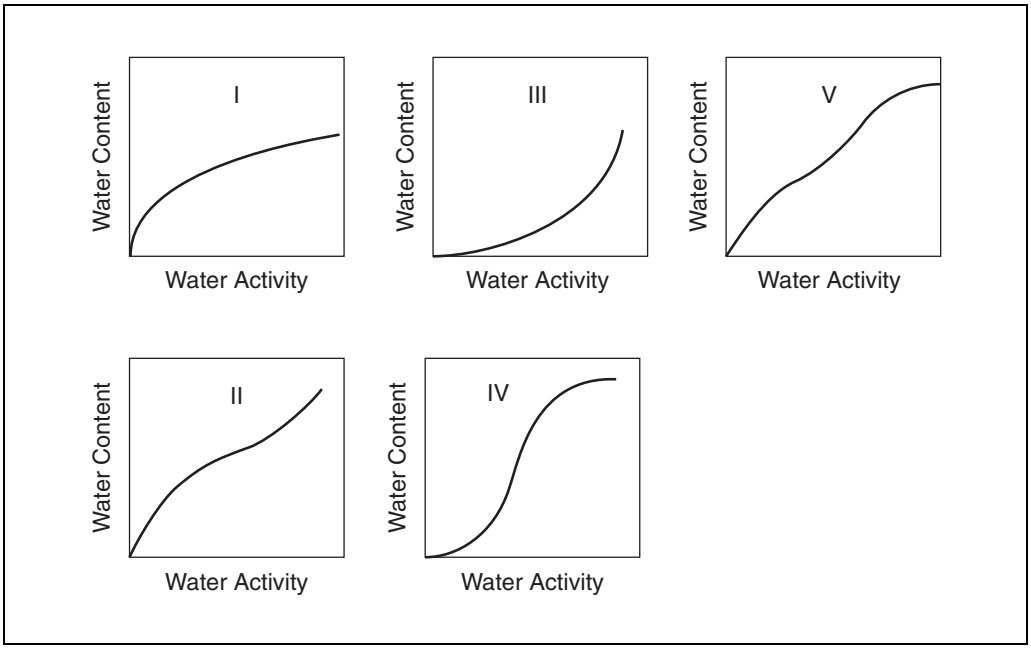

Figure A2.3.5 Five types of van der Waals adsorption isotherms proposed by Brunauer et al. (1940).

$$M_w = \frac{M_m Y K a_w}{(1 - K a_w)(1 - K a_w + Y K a_w)}$$

Equation A2.3.5

$$Y = Y_o \exp\left(\frac{\Delta H_C}{RT}\right)$$

Equation A2.3.6

$$K = K_o \exp\left(\frac{\Delta H_K}{RT}\right)$$

Equation A2.3.7

where M_m is the BET or GAB monolayer moisture (kilogram water/kilogram dry solid), B is a constant related to the net heat of sorption, ΔH_C and ΔH_K are heat of sorption functions. The most popular BET isotherm usually holds only water activities from ~0.05 to 0.45, but this gives a value of monolayer moisture content.

Time Considerations

Basic protocol

The salt solutions should be allowed to stand for a few days at the desired temperature before use. Several days, weeks, or even months may be required to establish equilibrium under static air conditions. Equilibrium can be enhanced with proper modification in the apparatus as discussed above.

Alternate protocol

This method is normally faster than the other one (see Basic Protocol) and usually takes 24 to 45 hr to reach equilibrium between salt and standard, or standard and sample.

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Direct Manometric Determination of Vapor Pressure

Water activity (a_w) is the ratio of the partial vapor pressure of water above a solution to that of pure water at the same specific temperature. It plays an important role in evaluating the microbial, chemical, and physical stability of foods during storage and processing. The vapor pressure in the headspace of a food sample can be measured directly by a manometer. A manometer has one or two transparent tubes and two liquid surfaces where pressure applied to the surface of one tube causes an elevation of the liquid surface in the other tube. The amount of elevation is read from a scale that is usually calibrated to read directly in pressure units. Makower and Myers (1943) were the first to use this method to measure vapor pressure exerted by food. Later, the method was improved, in terms of design features of the apparatus, by various scientists (Taylor, 1961; Labuza et al., 1972; Lewicki, 1987). Troller (1983), Lewicki (1989), and Zanoni et al. (1999) used a capacitance manometer instead of a U-tube manometer for the measurement of vapor pressure. Lewicki et al. (1978) showed that the precision and reproducibility of the method can be improved by the simultaneous measurement of the water vapor pressure and temperature of the food sample. The method is reviewed in detail by Rizvi (1995) and Rahman (1995).

Two procedures for measuring vapor pressure of a given sample are described in this unit. The Basic Protocol directly measures the vapor pressure above the sample inside the flask. The Alternate Protocol improves the measurements by incorporating the correction factor due to the change in volume that occurs when water vapor is eliminated from the air/water vapor mixture during desiccation.

DETERMINATION OF WATER ACTIVITY IN FOOD SAMPLES BY MANOMETRIC VAPOR PRESSURE MEASUREMENTS

BASIC PROTOCOL

The water activity of food samples can be estimated by direct measurement of the partial vapor pressure of water using a manometer. A simple schematic diagram is shown in Figure A2.4.1. A sample of unknown water activity is placed in the sample flask and sealed onto the apparatus. The air space in the apparatus is evacuated with the sample flask excluded from the system. The sample flask is connected with the evacuated air space and the space in the sample flask is evacuated. The stopcock across the manometer is closed and temperatures are read. The equilibrium manometer reading is recorded (h_1). The stopcock over the sample is closed and the air space is connected with the desiccant flask. The manometer reading in the legs is read to give h_2. The water activity of the sample is then calculated (Labuza et al., 1976) as:

$$a_w = \frac{[h_1 - h_2]\rho g}{P_w^v}$$

Equation A2.4.1

where P_w^v is vapor pressure (in Pa) of pure water at T°C, ρ is the density (kg/m^3) of manometric fluid, g is acceleration due to gravity (in m/sec^2), and h_1 and h_2 are manometer readings (height of the fluid in m).

Contributed by M.S. Rahman, S.S. Sablani, N. Guizani, T.P. Labuza, and P.P. Lewicki

Current Protocols in Food Analytical Chemistry (2001) A2.4.1-A2.4.6

Materials

10 to 50 g food sample
10 to 15 g desiccant ($CaSO_4$ or silica gel)
Vapor pressure manometer apparatus consisting, e.g., of sample flask, desiccant flask, U-tube, manometric fluid (such as Apiezon B, from Sigma), and vacuum pump (Fig. A2.4.1) or similar apparatus with capacitance manometer
Additional reagents and equipment for gravimetric (*UNIT A1.1*) or Karl Fischer (*UNIT A1.2*) determination of water

1. Place 10 to 50 g of sample in the sample flask and seal the flask onto the apparatus.
2. Fill another flask with 10 to 15 g $CaSO_4$ or silica gel desiccant.

   The advantage of silica gel is that it changes color from blue (dry state) to red (wet state).

3. Isolate the sample flask by closing stopcock 5 (refer to Fig. A2.4.1). Evacuate the system to a pressure <200 mmHg. Next, open stopcock 5 to evacuate the sample for 1 to 2 min.

   The lower the final pressure, the faster the equilibration time, and the more precise the estimation of a_w.

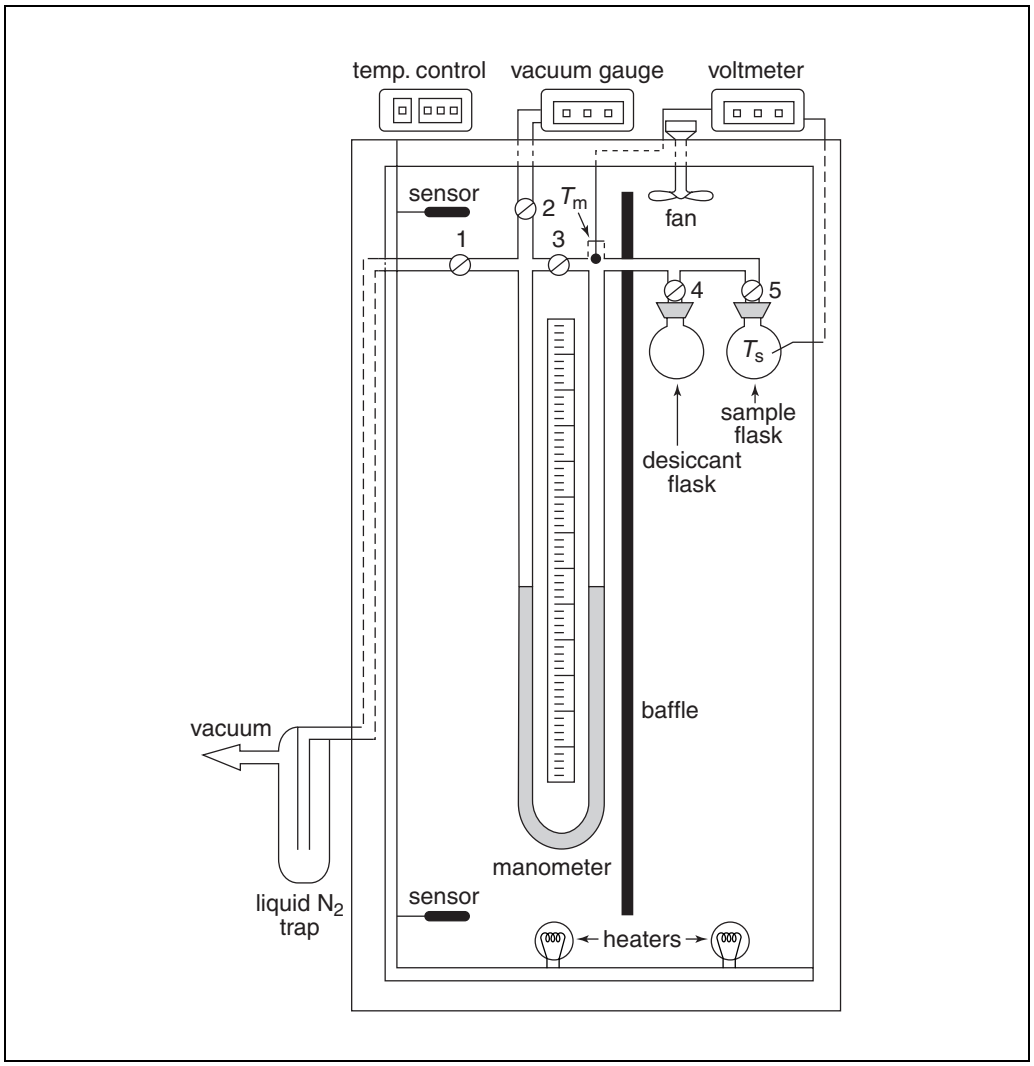

Figure A2.4.1 Schematic diagram of a thermostatted vapor pressure manometer apparatus. Reprinted from Rizvi (1995) with permission from Marcel Dekker.

4. Isolate the vacuum source and desiccant flask by closing stopcocks 1 and 4 (Fig. A2.4.1).
5. Allow the sample to equilibrate for 30 to 50 min, and then record the difference in manometer oil height (h_1 in m).

   This is the pressure in the headspace of the sample.
6. Exclude the sample flask by closing stopcock 5 and opening stopcock 4 of the desiccant flask.
7. After the water vapor is removed by sorption onto the desiccant, record the difference in manometer oil height (h_2 in m).

   This is the pressure exerted by volatiles and gases in the system.
8. Calculate the water activity, a_w, of the sample from h_1 and h_2 values using Equation A2.4.1.
9. If the temperature of the sample (T_s in K) and of the vapor space in the manometer (T_m in K) are different, then correct water activity using the following equation (Rizvi, 1995).

$$a_w = \frac{[h_1 - h_2]\rho g}{P_w^v} \frac{T_s}{T_m}$$

Equation A2.4.2

10. Measure the moisture content of the sample gravimetrically in an air or vacuum oven (*UNIT A1.1*) or by Karl Fischer titration (*UNIT A1.2*).

    Air drying at 105°C or vacuum drying at 70°C for >18 hr is usually used to determine the solid mass in the sample. The Karl Fisher technique is generally recommended, due to temperature abuse in the oven method. However, the oven method is widely used due to its simplicity and availability in most laboratories. The infrared moisture analyzer may also be used for quick determination of water content although it gives a different value from that obtained by the other two methods.

DETERMINATION OF WATER ACTIVITY CORRECTED FOR CHANGE IN VOLUME OF SYSTEM THAT OCCURS DURING DESICCATION

ALTERNATE PROTOCOL

This refined protocol uses a correction for the change in volume that occurs when water vapor is eliminated from the air/water vapor mixture when the desiccant flask is opened during desiccation. The precision and accuracy of the Basic Protocol can be significantly improved by taking this correction into account.

Additional Materials *(also see Basic Protocol)*

10 to 15 g P_2O_5

1. Place 10 to 15 g of P_2O_5 in both sample and desiccant flasks.
2. Open stopcocks 1, 3, and 5. Close stopcock 4 and evacuate the sample flask.

   Refer to Figure A2.4.1 for the location of all stopcocks and other features of the manometer.
3. Close stopcock 3 and note the manometric reading (h'_1).
4. Close stopcock 5, open stopcock 4, and obtain the manometric reading (h'_2).
5. Take manometric measurements (h_1 and h_2) of the sample (see Basic Protocol, steps 1 to 7).

6. Measure the void volumes V_4 and V_5 corresponding to the desiccant and the sample flasks.

   V_3 is the volume of the vapor pressure manometer between valves and the manometer liquid at zero reading.

7. Calculate a_w using the following equation, which takes into account the change in volume (Nunes et al., 1985; Lewicki, 1987):

$$a_w = \frac{[h_1 - Ch_2]\rho g n}{P_w^v}$$

Equation A2.4.3

where:

$$C = \frac{V_3 + V_4 + bh_2'}{V_3 + bh_1'}$$

Equation A2.4.4

and

$$n = \frac{273.15 + T_s}{273.15 + T_m}$$

Equation A2.4.5

and

$$b = \frac{\pi d^2}{80}$$

Equation A2.4.6

where d is the inner diameter of the glass tube (cm), and T_s and T_m are the temperatures of sample and manometer in °C.

8. Measure the moisture content of the sample (see Basic Protocol, step 10)

COMMENTARY

Background Information

The pressure exerted at equilibrium by the vapor above a liquid (or moist solid) contained in a closed system at a given temperature is known as the saturated or equilibrium vapor pressure. At equilibrium, the rate of liquid molecules evaporating and entering the vapor phase is considered to be equal to the rate of vapor molecules condensing into the liquid phase. The vapor pressure increases with the increase in temperature.

Water activity of food can be estimated by measuring the vapor pressure directly over it. Water activity is an extremely valuable tool for food scientists because of its usefulness in predicting food stability during storage, i.e., its tendency toward microbial, chemical, and physical deterioration. Most foods have a critical water activity below which the rate of quality loss is negligible.

The vapor pressure manometer (VPM) provides a means for rapid determination of water activity. The method was suggested by Makower and Myers (1943) to measure water vapor pressure exerted by food. This method was improved by Taylor (1961), Labuza et al. (1972), and Troller (1983). Sood and Heldman (1974), Labuza et al. (1976), and Lewicki et al. (1978) studied design features, accuracy, and precision of the VPM. Troller (1983) and Zanoni et al. (1999) discussed the design and setup of a water vapor pressure capacitance manometer.

The U-tube manometer has two transparent tubes connected together to form a U shape. Pressure is applied to one side of the tube, and the other side of the tube is normally open to

the atmosphere so that the amount of elevation can be read from a pressure scale engraved on the (later side of the) tube. In the capacitance type of manometer, a capacitor, consisting of two metal plates or conductors separated by an insulating material (dielectric), is used. The change in capacitance is calibrated with the pressure. The U-tube required is much larger in size than the capacitor used as a pressure sensor.

The accuracy of VPM measurements may depend upon the range of water activity being measured. Acott and Labuza (1975) gave an accuracy of 0.005 unit of a_w for $a_w < 0.85$ using this method. At higher values of a_w the accuracy is no better than 0.02 units of a_w because of temperature fluctuation (Labuza et al., 1976).

Lewicki et al. (1978) have shown that the accuracy of the measurement can be improved by taking into account differences between sample and headspace temperature. Troller (1983) and Nunes et al. (1985) showed that both accuracy and precision of the VPM improved significantly: 0.009 units of a_w in the a_w range 0.75 to 0.97. Lewicki (1987) demonstrated that design features of the VPM could also affect the accuracy and precision of the measured value. Although used as the standard method, the VPM is not suitable for materials that either contain large amounts of volatiles and bacteria or mold, or that are undergoing respiration processes (Rizvi, 1994).

Critical Parameters and Troubleshooting

The whole system should be maintained at a constant temperature. This can be achieved by immersing the sample holder in a constant-temperature water bath or by placing the whole system in a thermostatted cabinet. A correction factor needs to be used for the first case.

The ratio of the sample volume to headspace volume should be large enough (close to 0.5) to minimize changes in water activity due to loss of water by vaporization. In samples of higher water content the ratio of sample to headspace volume could be lower.

A low-density and low-vapor-pressure oil should be used as manometric fluid. Apiezon B (e.g., Sigma) manometric oil of density 866 kg/m^3 is generally recommended as manometric fluid.

The oil in the manometer may become contaminated with volatile compounds, and needs to be replaced from time to time (e.g., once every 2 months).

If the temperature of sample and the vapor space in the manometer are different, then water activity should be corrected (see Basic Protocol, step 9).

The desiccant should be replaced or regenerated by drying in an air/vacuum oven after each experiment.

Care should be taken to avoid foaming under vacuum. If excessive foam develops, this method may not be suitable.

Calibration must be done with a certain amount (10 to 15 g) of P_2O_5 placed in the sample flask, and the volume of food sample placed in the sample flask must be as close as possible to that of the P_2O_5.

Calibration needs to be done with same mass of desiccant P_2O_5. When desiccant is replaced, the mass of new desiccant ($CaSO_4$ or silica gel) must be the same as that used for calibration.

Anticipated Results

The experimental results provide water activity of a given sample at a specified temperature. The water activity measurements may range from 0.05 to 0.99. Isotherms can also be obtained by plotting water activity of a sample against its corresponding moisture content. The resultant isotherms can be modeled by empirical or theoretical equations as described in *UNIT A2.3*.

Time Considerations

The measurement of a_w (Basic Protocol) requires ~1 hr of equilibration time and does not require internal calibration. The calibration procedure (see Alternate Protocol) is also simple and straightforward, and can be done in 2 to 3 hr. The equilibration time in this protocol usually takes 1 hr.

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Measurement of Water Activity by Electronic Sensors

BASIC PROTOCOL

Water activity of foods is an important thermodynamic property affecting stability with respect to physical, chemical and microbiological changes. Water activity, a_w, is the ratio of the vapor pressure of water in a system, $(P^v_w)_{sy}$, to the vapor pressure of pure water, $(P^v)_w$, at the same temperature. It is equal to the equilibrium relative humidity (ERH) established in the surrounding air. Thus:

$$a_w = (P^v_w)_{sy}/(P^v)_w = \text{ERH}$$

Several instruments based on electronic sensors are commercially available for direct determination of water activity. This type of equipment is based on the measurement of the characteristic response of an electronic sensor in equilibrium with the air in a small chamber containing the sample in a plastic cup (usually with a volume of 15 ml). A schematic diagram of typical equipment is shown in Figure A2.5.1. Different types of sensor are commercially available (Rahman, 1995). Currently, most food laboratories are using sensor-type instruments, especially immobilized salt solution sensors.

In electrical resistance- or capacitance-type sensors, the conductivity or capacitance of a salt solution (usually lithium chloride) in equilibrium with the air is measured. The electrolytic sensor consists of a small hollow cylinder covered with a glass fiber tape impregnated with saturated lithium chloride solution. A spiral bifilar electrode is wound over the tape and a temperature sensor is mounted at the center of the cylinder. An alternating voltage is applied to the electrodes and a current is allowed to pass through the electrolyte. The resulting rise in temperature opposes the absorption of moisture by the lithium chloride, and the sensor rapidly reaches an equilibrium temperature at which the vapor pressure of the salt solution equals that of the air. Temperature is determined by a sensor at the core of the cylinder and a calibration chart is used to convert this to relative humidity. Hygroscopic organic polymer films are sometimes used instead of lithium chloride salt. Another type of sensor is the anodized aluminum sensor. This sensor

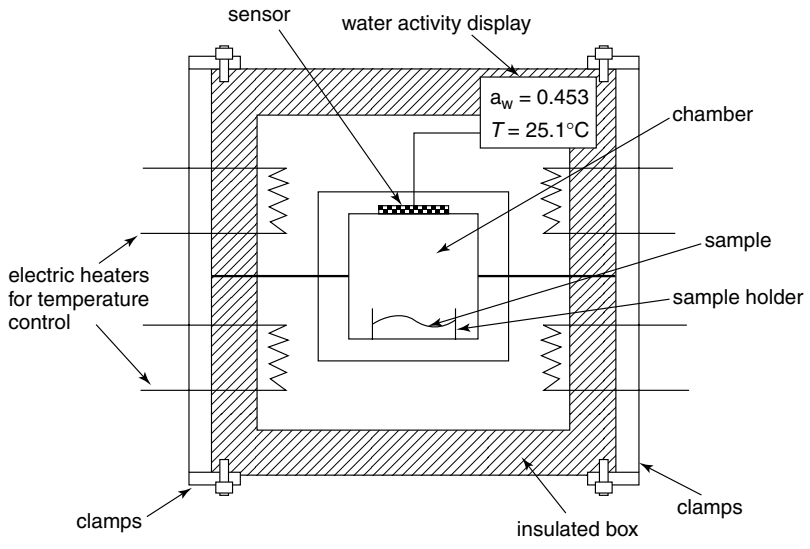

Figure A2.5.1 Schematic diagram of setup for water activity measurement by electronic sensor.

Contributed by M.S. Rahman and S.S. Sablani

Current Protocols in Food Analytical Chemistry (2002) A2.5.1-A2.5.4

consists of an aluminum strip that is anodized by a process that forms a porous oxide layer. A very thin coating of gold is then evaporated over this structure. The aluminum base and the gold layer form the two electrodes of what is essentially an aluminum oxide capacitor (Smith, 1971; ASHRAE, 1993; Rahman, 1995).

A multilaboratory, multi-instrument study of the use of electronic sensors applied to a cross-section of commodities and reference standards demonstrated that measurements of water activity by the described method (with instruments using immobilized salt solution sensors) can be made with an accuracy and precision within ±0.01 water activity unit, provided there are no interactions between the instrument and the commodity under investigation (Stoloff, 1978). Commodity-instrument interactions did exist with a number of products, especially those containing highly volatile compounds.

Materials

10 g saturated salt solution (Table A2.3.1 and Table A2.3.2) or standard supplied with the equipment from the manufacturer

5 g sample from bulk food, retaining its original state (e.g., solid with original structure liquid, or ground sample)

Instrumentation for measuring water activity by electronic sensors (American Instrument Company, Beckman Instruments, Nova Sina AG, and Rotronic AG)

Plastic sample holder with lid (diameter ~4.0 cm; height ~1.0 cm; usually supplied with instrument)

Calibrate instrument

1. Turn on the instrument and set it at a specified temperature (that at which water activity of sample needs to be measured), and then keep it at that temperature for at least 20 to 30 min to allow stabilization.

   Commercial equipment is usually available for measurements between 25° and 80°C. Calibration standards and samples must be measured at the same known temperature.

2. To prepare a calibration curve, choose at least five different saturated salt solutions that have water activities spread over the range of 0.11 to 0.99 at the temperature chosen for the sample.

   Calibration is performed by measuring the water activities of various saturated salt solutions and comparing them to values in the literature or values calculated from Table A2.3.2. Literature values at a range of temperatures are found in Table A2.3.1. Values at intermediate temperatures can be predicted from the equations in Table A2.3.2.

   Saturated salt solutions should contain excess salt (i.e., a saturated salt slurry) to ensure that the solution is completely saturated.

3. Place 10 g of each saturated salt solution in a plastic container (usually supplied with the equipment).

4. Place the plastic containers with salt solution in the equipment chamber one at a time for equilibration. Record instrument response at $t = 0$.

   Calibration should be done at the temperature selected in step 1.

5. Continue taking instrument readings of the samples, staggering them so that each is measured at 15-min intervals. Continue until equilibrium is reached (up to 120 min, if necessary).

   Two consecutive readings at 15-min intervals that vary by <0.01 a_w unit are evidence of adequate equilibration.

6. Record the water activity displayed by the equipment and calculate the correction factor by using the value given in Table A2.3.1 for the solution.

   This is the correction factor for the specific instrument.

7. Prepare a calibration curve by plotting the instrument readouts for the five (or more) saturated salt solutions against their actual water activities given in Table A2.3.1.

   It is good to calibrate the equipment frequently, for example, every week. If the test sample contains high volatile content, it is preferable that calibration be performed after each experiment. Make all measurements within the range of calibration points, and do not extrapolate the calibration line. Make all measurements in the same direction of change (i.e., from lower to higher water activity or higher to lower water activity).

Measure water activity

8. Place 5 g of sample in a plastic container and then place the plastic container with sample in the equipment chamber for equilibration.

9. Wait until the reading does not change by more than 0.01 a_w unit.

   Equilibration usually takes 15 to 30 min depending on the type of food and the measurement temperature.

10. Record the water activity reading. Remove the plastic container from the chamber and keep it sealed with its plastic lid until moisture content is measured.

11. Determine the moisture content by gravimetric or other methods (*UNITS A1.1 & A1.2*).

COMMENTARY

Background Information

The isopiestic and manometric methods (*UNITS A2.3 & A2.4*) for determination of water activity have the limitation of being dependent on fixed laboratory equipment. The electronic-type sensors have advantages of portability, speed, and simplicity of measurement. The characteristics of a sensor depend upon the manufacturer and each instrument must be calibrated separately. The anodized sensors have advantages of ruggedness, small dimensions, and fast response, as well as freedom from large temperature coefficients and less susceptibility to contamination of the lithium chloride conductivity sensors (Smith, 1971).

Equipment based on the change in electrical conductivity of immobilized salt solution is available from American Instrument Company, Beckman Instruments, Nova Sina AG, and Rotronic AG. Equipment based on change in electrical capacitance of polymer thin films is available from WeatherMeasure and General Eastern Corporation. The approximate price range for water activity measurement instruments based on electronic sensors is $6000 to $8000.

Critical Parameters

Specific problems arising from the use of a sensor for water activity measurement are equilibration time, the calibration of the sensors, and the influence of nonuniform temperature on the values measured in saturated salt solutions and food products. Each sensor must be calibrated separately. The performance of a sensor is subject to change on aging and contamination of the element by foreign particles. The contamination of the sensor by volatile components can cause erroneous results, and thus calibration is important. A mechanical or chemical filter is available to protect the sensor from contamination. The Sina-scope sensor (Nova Sina AG) is equipped with a mechanical filter to protect it from dust, oil, and water vapor condensation. A chemical filter can also be used to protect the sensor from chlorine, formaldehyde, ammonia, sulfur dioxide, hydrogen sulfide, amino acids, hydrocarbons, and oil droplets (Labuza et al., 1976). Large differences in temperature between sample and sensor should always be avoided because of possible condensation. Sample temperature must be known.

Long-term use of a sensor at higher temperatures damages or reduces the life of the sensor by reducing its precision and accuracy. Thus, a large number of measurements at high temperature should be avoided, to increase the life of the sensor. It is difficult to find a sensor that will operate at both very low and very high tempera-

tures (e.g., both below 0°C and above 100°C; Wiederhold, 1987).

Equilibrium times obtained in measurement on various food products have ranged from a minute to several hours. Longer equilibration time is required for lower water activity measurement, especially water activity below 0.7; however, very long equilibration time (e.g., more than 1 hr) for water activity higher than 0.9 can cause saturation of the sensor. A saturated sensor always gives water activity equal to 1.0. In this case, small sample size is preferred. Stekelenburg and Labots (1991) found that the equilibration time required for various products to reach a constant water activity value (a change of <0.01 unit) increased at higher water activity, and mentioned that the following precautions must be taken for reliable measurement: (1) the water activity value should be taken when the reading (0.001 unit) has been constant for 10 min, (2) the humidity sensors should be calibrated regularly to compensate for drift, (3) a separate calibration curve should be made for each sensor, and (4) sensors should be calibrated at the same temperature at which the samples are measured. Differences in temperature between sample and sensor should be avoided because of possible formation of condensate on the sensor. Labuza et al. (1976) found that the equilibration time varied from 20 min to 24 hr depending on the humidity range and food materials. They found that readings did not change significantly after 20 or 30 min; therefore, 30-min readings were recommended.

Anticipated Results

Water activity measurement by electronic sensor gives the water activity value within the range 0 to 1 (up to three decimal points) as a function of sample water content and measurement temperature (25° to 50°C).

Time Considerations

In this method, time is required for preparing the saturated salt slurry (1 hr) and the sample (30 min), as well as for temperature equilibration (30 min) and determining the moisture content (30 min). Once standard saturated salt slurries are prepared, they can be used for at least a month unless microflora growth is observed. A saturated salt solution is indicated by the presence of a salt crystal layer at the bottom of the cup. Usually, 30 min equilibrium time is found to be adequate for a sample; however, in some cases, a couple of hours may be needed.

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Key References

Labuza et al., 1976. See above.

Describes sources of error and details of measurement procedure.

Rahman, 1995. See above.

Provides general descriptions of types of sensors and their applicability and limitations.

Contributed by M.S. Rahman and S.S. Sablani
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The Colorimetric Detection and Quantitation of Total Protein

Protein quantification is an important step for handling protein samples for isolation and characterization, and is a prerequisite step before submitting proteins for chromatographic, electrophoretic, or immunochemical analysis and separation. The methods included in this unit are colorimetric measurements, whose procedures are faster, simpler, and less laborious than those based on estimation of total nitrogen content (*UNIT B1.2*).

This unit describes four of the most commonly used total protein assay methods. Three of the four are copper-based assays to quantitate total protein: the Lowry method (see Basic Protocol 1 and Alternate Protocols 1 and 2), the bicinchoninic acid assay (BCA; see Basic Protocol 2 and Alternate Protocols 3 and 4), and the biuret method (see Basic Protocol 3 and Alternate Protocol 5). The fourth is the Coomassie dye binding or Bradford assay (see Basic Protocol 4 and Alternate Protocols 6 and 7), which is included as a simple and sensitive assay, although it sometimes gives a variable response depending on how well or how poorly the protein binds the dye in acidic pH. A protein assay method should be chosen based on the sensitivity and accuracy of method as well as the condition of the sample to be analyzed.

STRATEGIC PLANNING

Colorimetric Protein Assays

The four colorimetric methods for the detection and quantitation presented in this unit have withstood the test of time. They are all well-characterized robust assays that consistently work well. The methods were introduced over the past 15 to 50 years. They collectively represent the state of the art for colorimetric detection and quantitation of total proteins in the microgram to milligram range.

When confronted with the need to determine the total protein concentration of a sample, one of the first issues to consider is selection of a protein assay method. The choice among the available protein assays usually is made based upon consideration of the compatibility of the method with the samples to be assayed. The objective is to select a method that requires the least manipulation or pretreatment of the samples due to the presence of substances that may interfere. If the total protein concentration in the samples is high (i.e., in the range of 5 to 160 mg/ml), the biuret total protein reagent is the best choice. If the total protein concentration in the samples is low (i.e., in the range of 1 to 2000 μg/ml), then any one of the other three (i.e., the Lowry, the Coomassie plus, or the BCA method) would be suitable. If the sample contains reducing agents or copper-chelating reagents, the Coomassie Plus Protein Assay Reagent (Pierce) would be the best choice. If the sample contains one or more detergents (at concentrations up to 5%), the BCA protein assay reagent is the best choice.

Sometimes the sample contains substances that make it incompatible with any of the protein assay methods. In those cases, some pretreatment of the sample is necessary.

Each method has its advantages and disadvantages. No one method can be considered to be the ideal or best protein assay method. Because of this, most researchers keep more than one type of protein assay reagent available in their lab.

Contributed by Randall I. Krohn

Current Protocols in Food Analytical Chemistry (2001) B1.1.1-B1.1.28

Selection of the Protein Standard

The selection of a protein standard is potentially *the* greatest source of error in any protein assay. Of course, the best choice for a standard is a highly purified version of the predominate protein found in the samples. This is not always possible nor always necessary. In some cases, all that is needed is a rough estimate of the total protein concentration in the sample. For example, in the early stages of purifying a protein, identifying which fractions contain the most protein may be all that is required. If a highly purified version of the protein of interest is not available or it is too expensive to use as the standard, the alternative is to choose a protein that will produce a very similar color response curve with the selected protein assay method.

For general protein assay work, bovine serum albumin (BSA) works well as the choice for a protein standard, because it is widely available in high purity and relatively inexpensive. Although it is a mixture containing several immunoglobulins, bovine gamma globulin (BGG) is also a good choice for a standard when determining the concentration of antibodies, since BGG produces a color response curve that is very similar to that of immunoglobulin G (IgG).

For greatest accuracy of the estimates of the total protein concentration in unknown samples, it is essential to include a standard curve in each run. This is particularly true for the protein assay methods that produce nonlinear standard curves (e.g., Lowry method, Coomassie dye–binding method). The decision about the number of standards used to define the standard curve and the number of replicates to be done on each standard depends upon the degree of nonlinearity in the standard curve and the degree of accuracy required of the results. In general, fewer points are needed to construct a standard curve if the color response curve is linear. For assays done in test tubes, duplicates are sufficient; however, triplicates are recommended for assays performed in microtiter plates due to the increased error associated with microtiter plates and microtiter plate readers.

Preparation of the Samples

Before a sample can be analyzed for total protein content, it must be solubilized, usually in a buffered aqueous solution. The entire process is usually done in the cold, with additional precautions taken to inhibit microbial growth or to avoid casual contamination of the sample by foreign debris such as hair, skin, or body oils. When working with tissues, cells, or solids such as food products, the first step of the solubilization process is usually disruption of the sample's cellular structure by grinding and/or sonication, or by the use of specially designed reagents containing surfactants to lyse the cells (i.e., the "POP-PERS" line of products, available from Pierce). This is done in a cold aqueous buffer containing one or more surfactants (to aid the solubilization of the membrane-bound proteins), one or more biocides (to prevent microbial growth), and protease inhibitors (to minimize or prevent digestion of the proteins into peptide fragments by endogenous proteases). After filtration or centrifugation (to remove the cellular debris), additional steps such as sterile filtration, removal of lipids, or further purification of the protein of interest from the other sample components may be necessary.

Calculation of the Results

If calculating the protein concentrations manually, it is best to use point-to-point interpolation. This is especially true if the standard curve is nonlinear. Point-to-point interpolation refers to a method of calculating the results for each sample using the equation for a linear regression line obtained from just two points on the standard curve. The first point is the standard that has an absorbance just below that of the sample and the second point is the standard that has an absorbance just above that of the sample. In this way, the

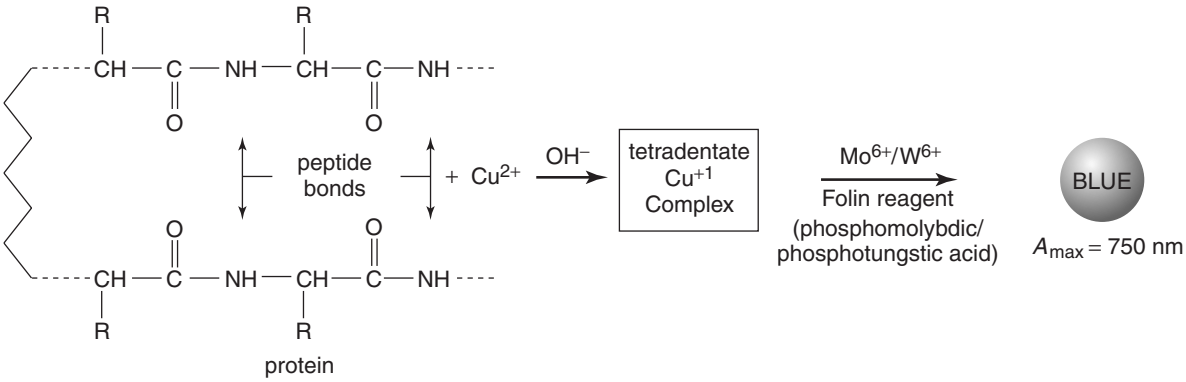

Figure B1.1.1 The reaction schematic for the Lowry Protein Assay.

concentration of each sample is calculated from the most appropriate section of the whole standard curve. The average total protein concentration for each sample is determined from the average of its replicates. If multiple dilutions of each sample have been run, the results for the dilutions that fall within the most linear portion of the working range are averaged.

If using a computer program, use a quadratic curve fit for the nonlinear standard curve to calculate the protein concentration of the samples. If the standard curve is linear or if the absorbance readings for the samples fall within the linear portion of the standard curve, the total protein concentrations of the samples can be estimated using the linear regression equation.

Most software programs will allow the experimenter to construct and print a graph of the standard curve as well as calculate the protein concentration for each sample and display statistics for the replicates. Typically, the statistics displayed will include the average of the absorbance readings (or the average of the calculated protein concentrations), the standard deviation (SD), and the coefficient of variation (CV) for each standard or sample. If multiple dilutions of each sample have been run, average the results for the dilutions that fall in the most linear portion of the working range.

THE LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

BASIC PROTOCOL 1

In 1951, Oliver H. Lowry introduced this colorimetric total protein assay method. It offered a significant improvement over previous protein assays, and his paper became one of the most cited references in the life-science literature (Lowry, 1951). The Lowry assay is easy to perform, since the incubations are done at room temperature and the assay is sensitive enough to allow the detection of total protein in the low microgram per milliliter range. It is one of three copper chelation chemistry–based methods presented in this unit. Essentially, the Lowry protein assay is an enhanced biuret assay (see Basic Protocol 3). After a short incubation, Lowry's reagent C (Folin phenol) is added for enhanced color development (Fig. B1.1.1). The Lowry assay requires fresh (daily) preparation of two reagents and a meticulously timed incubation step. The two reagents are combined just before use to make a buffered alkaline cupric sulfate working solution. The addition of sodium dodecyl sulfate (SDS) to Lowry's reagent D (i.e., Lowry's reagent D′) allows the method to be used with samples that contain detergents.

Materials

Standard protein: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample(s)
Lowry's reagents C and D or D′ (see recipes)

1. Dispense 0 to 100 µl standard protein to appropriately labeled tubes and bring the total volume to 100 µl with sample buffer or solvent to prepare a dilution series from 10 to 100 µg.

   These concentrations of albumin should produce A_{750} readings from ~0.10 to 1.0 AU in 1-cm cuvettes.

2. Dispense ≤100 µl protein sample(s) to separate labeled tubes and adjust the final volume to 100 µl using the same buffer or solvent used to prepare the sample.

3. Add 1 ml reagent D (or reagent D′) to each of the standards and unknown samples. Vortex the tubes immediately to develop optimum color. Incubate for precisely 10 min at room temperature.

   If samples contain detergent, use reagent D′ to eliminate the interference associated with up to 1% of various detergents. If a precipitate forms in reagent D′, warm the reagent and vortex before addition.

4. While mixing, add 0.1 ml reagent C. Vortex the tubes immediately. Incubate for 30 min at room temperature.

5. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

   NOTE: *Read samples within 10 min, as samples continue to develop color. Samples incubated longer than 60 min should be discarded.*

   If the absorbance reading of the sample is higher than that of the highest concentration of standard, dilute the sample with buffer and repeat the procedure on the diluted sample.

6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in milligrams per milliliter.

7. Determine sample protein concentration by interpolating from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 1

MODIFIED LOWRY PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEINS

Preformulated, stabilized, modified versions of the Lowry reagent are now commercially available from Pierce (the Modified Lowry Protein Assay Reagent) or from Bio-Rad (the DC Protein Assay). The assay can be performed in test tubes or a microtiter plate (see Alternate Protocol 2). The working range of this assay is 1 to 1500 µg/ml if the Pierce reagent is used, or 200 to 1400 µg/ml if the Bio-Rad reagent is used. Table B1.1.1 is a brief troubleshooting guide for this technique.

Additional Materials *(also see Basic Protocol 1)*

Modified Lowry Protein Assay Kit (Pierce) containing:
2 mg/ml BSA in 0.9% (w/v) NaCl/0.05% (w/v) sodium azide
2 N Folin-Ciocalteu reagent: dilute fresh to 1 N
Modified Lowry's Reagent

Table B1.1.1 Troubleshooting Guide for the Modified Lowry Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a chelating agent (e.g., EDTA, EGTA)	Dialyze or dilute the sample
		Precipitate the protein with TCA and dissolve the pellet in modified Lowry reagent
Blank A_{750} is normal, but standards show less color than expected	Sample changed the pH of the reagent	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 750 nm
Precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
	Sample contains potassium ions	Precipitate the protein with TCA, dissolve the pellet in Modified Lowry Reagent
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with TCA, dissolve pellet in Modified Lowry Reagent
Need to read color at a different wavelength	Colorimeter does not have 750-nm filter	Color may be read at any wavelength between 650 nm and 750 nm

1. Prepare a dilution series of 2 mg/ml BSA (e.g., the standard provided in the Modified Lowry Protein Assay Kit) in buffer to cover the range 2.0 to 1500 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. In duplicate, add 200 µl diluted standard, sample, or buffer (blank) into appropriately labeled test tubes.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. At 15-sec intervals, add 1.0 ml Modified Lowry's Reagent to each of the tubes. Vortex 2 to 3 sec to mix the contents of the tube and incubate at room temperature for *exactly* 10 min.

4. At the end of the first tube's 10-min incubation, add 100 µl freshly diluted 1 N Folin-Ciocalteu reagent (freshly diluted from a 2 N stock). Immediately vortex the tube for 2 to 3 sec. Continue to maintain the 15-sec intervals from step 3 for addition of the reagent to the remaining tubes.

5. Allow each of the tubes to incubate at room temperature for 30 min.

6. Measure the color at 750 nm (A_{750}) on a spectrophotometer zeroed with deionized water.

7. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each BSA standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure B1.1.2.

8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Determine the average total protein concentration for each sample from the average of its replicates.

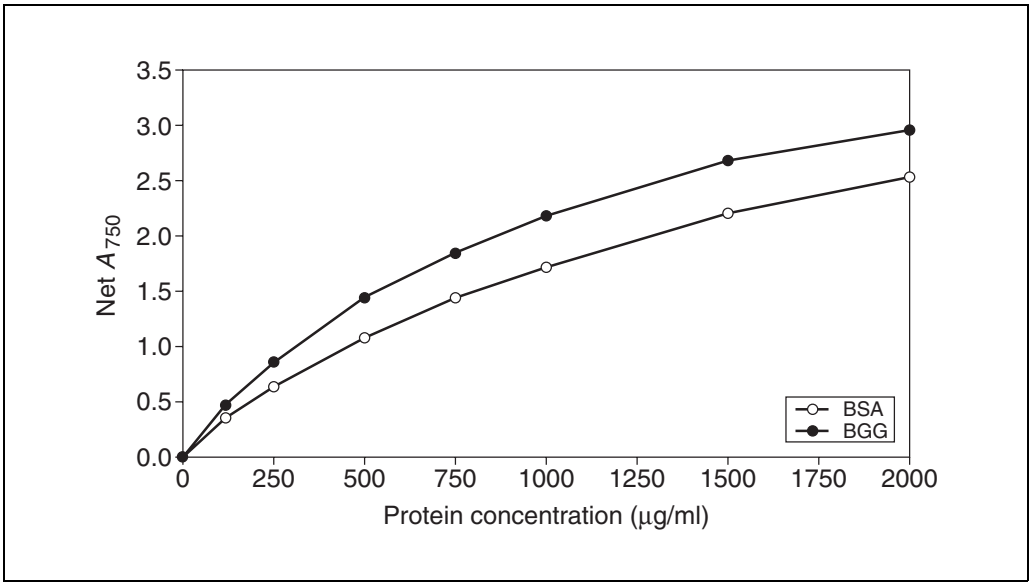

Figure B1.1.2 Graph of the color response curves obtained with Pierce's Modified Lowry Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 750 nm in a Hitachi U-2000 spectrophotometer.

ALTERNATE PROTOCOL 2

MICROTITER PLATE MODIFIED LOWRY ASSAY

The modified Lowry assay can also be done in a 96-well microtiter plate format. The assay has a working range of 1 to 1500 µg/ml.

Additional Materials *(also see Alternate Protocol 1)*

Microtiter plate and cover or tape seals
200-µl multichannel pipettor
Microtiter plate reader for 750 nm

1. Draw a template for placement of samples and standards on the microtiter plate.
2. Add 40 µl of each diluted BSA standard (see Alternate Protocol 1, step 1), sample, or diluent (blank) to the appropriate wells of a 96-well plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

3. Using a multichannel pipettor, quickly add 200 µl Modified Lowry's Reagent to each of the wells. Mix immediately on a plate mixer for 30 sec.
4. Allow the plate to incubate at room temperature for *exactly* 10 min.
5. Using a multichannel pipettor, quickly add 20 µl freshly diluted 1 N Folin-Ciocalteu reagent to each well. Immediately mix on a plate mixer for 30 sec.
6. Cover the plate (to prevent evaporation) and incubate 30 min at room temperature.
7. Mix the plate again and measure the color (absorbance) of each well in a microtiter plate reader at 750 nm.
8. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for each standard versus its protein concentration in micrograms per milliliter.
9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

THE BICINCHONINIC ACID (BCA) FOR DETERMINATION OF TOTAL PROTEIN

Smith et al. (1985) introduced the bicinchoninic acid (BCA) protein assay reagent. In one sense, it is a modification of the Lowry protein assay reagent. The mechanism of color formation with protein for the BCA protein assay reagent is similar to that of the Lowry reagent, but there are several significant differences. The BCA protein assay reagent combines the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (i.e., the biuret reaction; see Basic Protocol 3) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The purple-colored reaction product of this method is formed by the chelation of two molecules of BCA with one cuprous ion (Fig. B1.1.3). The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The primary advantage of the BCA protein assay reagent is that most surfactants, even if present in the sample at concentrations up to 5% (v/v), are compatible with this method. Table B1.1.2 is a brief troubleshooting guide for this technique.

Materials

Protein standard: 2 mg/ml BSA (see recipe)
Sample buffer or solvent
Protein sample
BCA working reagent: mix 100 parts BCA reagent A with 2 parts reagent B (see recipes for each reagent)

1. Prepare a dilution series of 2 mg/ml BSA in sample buffer or diluent to cover a range from 125 to 2000 µg/ml.
2. Add 100 µl sample, diluted standard, or buffer (blank) into appropriately labeled tubes.
3. Add 2 ml BCA working reagent mix to each tube. Vortex immediately.
4. Incubate samples and standards for 30 min at 37°C, then cool to room temperature.
5. Measure the color at 562 nm (A_{562}) on a spectrophotometer zeroed with deionized water.

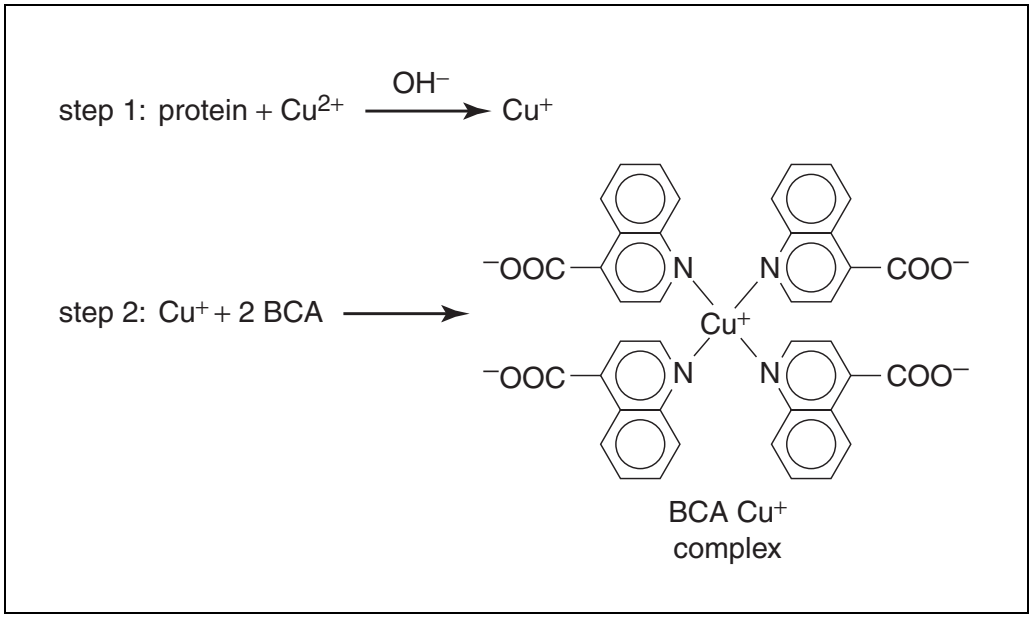

Figure B1.1.3 The reaction schematic for the BCA Protein Assay.

Table B1.1.2 Troubleshooting Guide for BCA Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains a copper chelating agent	Dialyze or dilute the sample
		Increase the copper concentration in the working reagent (use 48 parts reagent A and 2 parts reagent B)
Blank A_{562} is normal, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze or dilute the sample
	Color measured at the wrong wavelength	Measure the color at 562 nm
Color of samples appear darker than expected	Protein concentration is too high	Dilute the sample
	Sample contains lipids or lipoproteins	Add 2% (w/v) SDS to the sample to eliminate interference from lipids
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample
	Sample contains a thiol	Precipitate the protein with trichloroacetic acid (TCA) and deoxycholate (DOC), dissolve pellet in BCA working reagent
	Sample contains biogenic amines (catecholamines)	Treat the sample with iodoacetamide (for thiols)
Need to read color at a different wavelength	Colorimeter does not have 562-nm filter	Color may be read at any wavelength between 550 nm and 570 nm

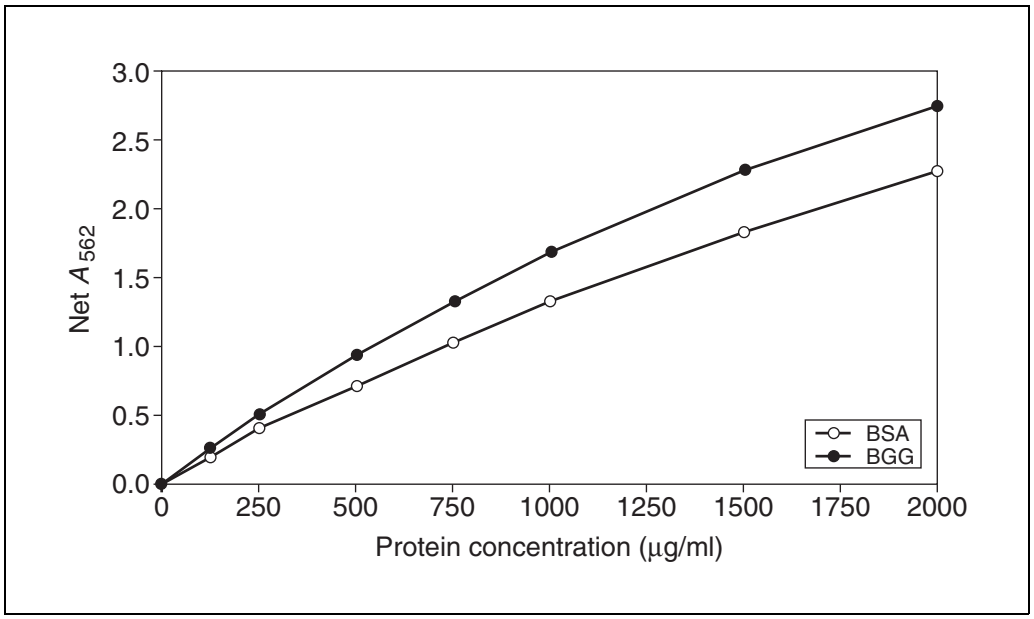

Figure B1.1.4 Graph of the color response curves obtained with Pierce's BCA Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 562 nm in a Hitachi U-2000 spectrophotometer.

6. Plot a standard curve by graphing the average net or blank-corrected A_{750} values for the standards versus protein concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure B1.1.4.

7. Determine the protein concentration of the sample by interpolation from the plot (see Strategic Planning).

USING KITS FOR BCA MEASUREMENTS OF TOTAL PROTEIN

ALTERNATE PROTOCOL 3

Preformulated versions of the BCA reagent are now commercially available from Pierce (BCA Protein Assay Reagent) or Sigma (Bicinchoninic Acid Kit for Protein Determination). This assay can be performed in test tubes or microtiter plates (see Alternate Protocol 4), and has a working range of 20 to 2000 µg/ml.

Additional Materials

BCA Protein Assay Reagent Kit (Pierce) or Bicinchoninic Acid Kit (Sigma) containing:
2 mg/ml BSA in 0.9% NaCl/0.05% sodium azide (also see recipe)
BCA reagent A (also see recipe)
BCA reagent B (also see recipe)
37°C water bath

1. Prepare a dilution series of BSA standard in buffer to cover the range 125 to 2000 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Prepare sufficient BCA working reagent (a minimum of 2 ml/tube or 20 ml/96-well microtiter plate) by adding 2 parts BCA reagent B to 100 parts of BCA reagent A.

   After mixing, the BCA working reagent is clear and apple green in color.

3. In duplicate, add 100 µl standard, sample, or buffer (blank) into appropriately labeled test tubes.

4. Add 2.0 ml BCA working reagent to each tube. Mix well by vortexing each tube 2 to 3 sec.

5a. *Standard tube protocol:* Incubate all tubes in a 37°C water bath for 30 min.

5b. *Enhanced assay protocol:* Alternatively, incubate all tubes in a 60°C water bath for 30 min.

   Increasing the incubation temperature to 60°C lowers the minimum detection level to 5 µg/ml and narrows the working range of the assay to a maximum of 250 µg/ml. This is known as the enhanced BCA assay.

6. After incubation, cool all tubes to room temperature.

   NOTE: *Since the BCA reagent does not reach a true end point, color development will continue even after cooling to room temperature; however, the rate of color development is very slow after cooling to room temperature, so no significant error is introduced if the A_{562} readings of all the tubes can be read within ~10 min.*

7. Before reading, mix each tube again and measure the amount of color produced in each tube with a spectrophotometer at 562 nm (A_{562}) versus deionized water.

8. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for the standards versus protein concentration in micrograms per milliliter.

9. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average sample concentration from its replicates.

ALTERNATE PROTOCOL 4

MICROTITER PLATE ASSAY FOR BCA MEASUREMENT OF TOTAL PROTEIN

BCA assays can be run in 96-well microtiter plates. The assay has a working range of 125 to 2000 µg/ml.

Additional Materials *(also see Alternate Protocol 3)*

96-well microtiter plate with cover or tape seal
200-µl multichannel pipettor
Microtiter plate shaker
37°C dry-heat incubator
Microtiter plate reader

1. Draw a template for planning samples and standards on a microtiter plate.

   Blanks, standards, and samples should be prepared in triplicate.

2. Add 10 µl of each diluted BSA standard (see Alternate Protocol 3, step 1), sample, or diluent (blank) to the appropriate wells.
3. Using a multichannel pipettor, add 200 µl BCA working reagent (see Alternate Protocol 3, step 2) to each well. Mix well on a microtiter plate shaker for 30 sec.
4. Cover the plate and incubate in a 37°C dry-heat incubator for 30 min.
5. After incubation, allow the plate to cool to room temperature.
6. Mix the plate again, remove the plate cover and measure the color in each well of the plate at 562 nm (A_{562}) in a microtiter plate reader.
7. Plot a standard curve by graphing the average net or blank-corrected A_{562} values for each BSA standard versus its concentration in micrograms per milliliter.
8. Determine the sample concentration by interpolating from the plot (see Strategic Planning). Calculate the average total protein concentration for each sample from the average of its replicates.

BASIC PROTOCOL 3

THE BIURET ASSAY FOR DETERMINING TOTAL PROTEIN

All proteins are composed of amino acids joined by peptide bonds in a linear sequence. There are ~20 naturally occurring amino acids found in proteins. The amino acids are joined to each other by peptide bonds formed by a condensation reaction that occurs between the terminal amine of one amino acid and the carboxyl end of the next. Peptides containing three or more amino acid residues will form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. A similar colored chelate complex forms with the organic compound biuret (NH_2–CO–NH–CO–NH_2) and the cupric ion. The reaction in which a colored chelation complex is formed with peptide bonds in the presence of an alkaline cupric sulfate solution became known as the biuret reaction (Fig. B1.1.5). Thus, the biuret protein assay reagent gets its name from the above reaction even though it does not actually contain the organic compound biuret. Single amino acids or dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce the light-blue to violet complex that absorbs light at 540 nm. One cupric ion forms the colored coordinate complex with 4 to 6 nearby peptides bonds. The intensity of the color produced is proportional to the

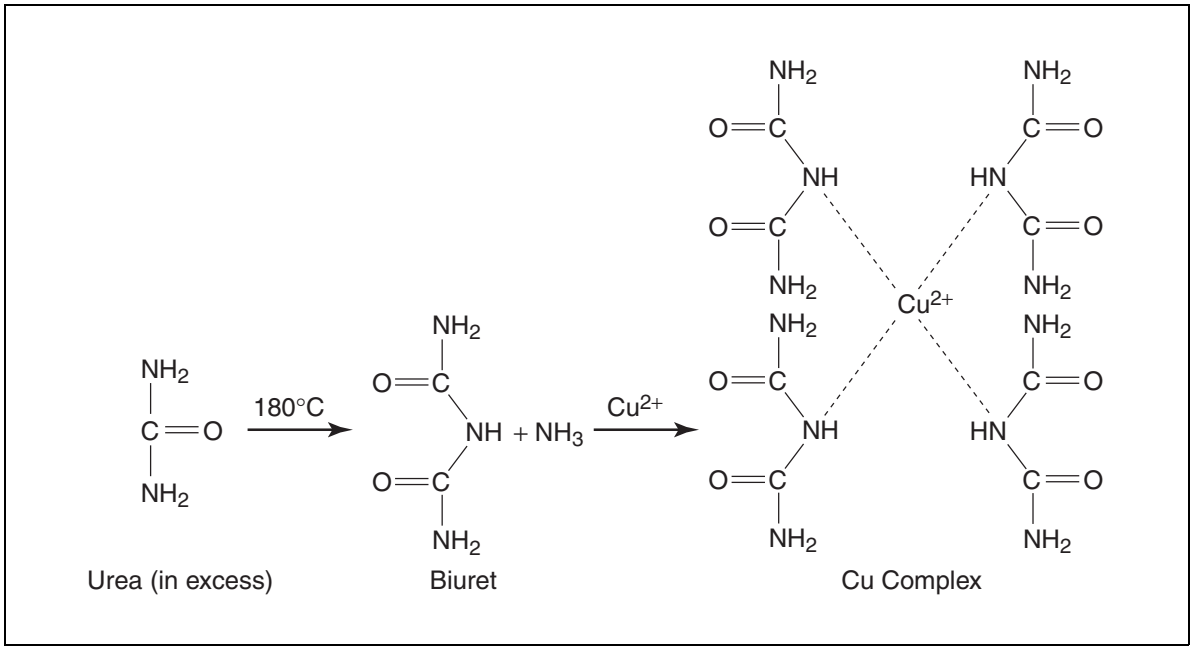

Figure B1.1.5 The schematic of the Biuret reaction.

Table B1.1.3 Troubleshooting Guide for Biuret Protein Assay

Problem	Possible cause	Solution
No color in any tubes	Sample contains copper chelating agent	Dialyze or dilute the sample
Blank A_{540} is normal, but standards show less color than expected	Color measured at the wrong wavelength	Measure the color at 540 nm
All tubes (including the blank) are dark purple	Sample contains a reducing agent	Dialyze or dilute the sample

number of peptide bonds participating in the reaction. Thus, the biuret reaction is the basis for a simple and rapid colorimetric method of quantitatively determining total protein concentration.

Because the working range for the Biuret assay is from 5 to 160 mg/ml, the Biuret reagent has found utility in the clinical laboratories for the quantitation of total protein in serum. The formulation employed in the Biuret total protein reagent (Sigma Diagnostics) was developed by Doumas et al. (1981) as a candidate reference method for the determination of serum total protein in the clinical lab. Using Sigma's Biuret reagent, the expected range for total protein in serum is from 63 to 83 mg/ml. Bilirubin, lipids, hemoglobin, and dextran are known to interfere in the Biuret assay for total serum protein. Outside of this application, other copper chelating agents such as EDTA, EGTA, citrate, Tris, iminodiacetic acid, and nitrilotriacetic acid will interfere. The working range of this assay is 5 to 160 mg/ml. Table B1.1.3 is a brief troubleshooting guide for this technique.

Materials

Standard protein (see Commentary)

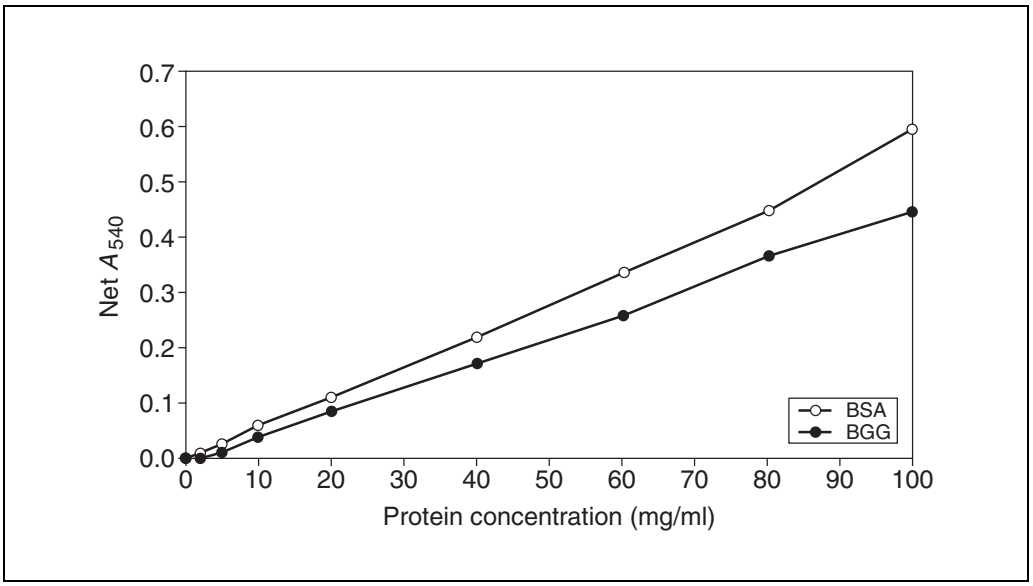

Figure B1.1.6 Graph of the color response curves obtained with Sigma's Biuret Total Protein Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 540 nm in a Hitachi U-2000 spectrophotometer.

Sample (unknown) protein
Biuret total protein reagent (Sigma Diagnostics; also see recipe)

1. Select a protein to use as the standard (see Strategic Planning) and prepare a dilution series with buffer to cover the range 10 to 160 mg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the standard that was used with the samples.

2. In duplicate, add 20 µl standard, sample, or diluent (blank) to appropriately labeled test tubes.
3. Add 1.0 ml biuret reagent to each tube. Mix well by vortexing 2 to 3 sec.
4. Incubate tubes at ambient room temperature (18° to 26°C) for 10 min.
5. Measure the color of each tube with a spectrophotometer at 540 nm (A_{540}). Compare to the blank.
6. Plot a standard curve by graphing the blank-corrected A_{540} values for the standards versus protein concentration in milligrams per milliliter.

   Example color response curves for BSA and BGG are shown in Figure B1.1.6.

7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

MICROTITER PLATE BIURET ASSAY FOR TOTAL PROTEIN

ALTERNATE PROTOCOL 5

The biuret assay can be performed in a 96-well microtiter plate and has a working concentration of 10 to 160 mg/ml.

Additional Materials *(see Basic Protocol 3)*

96-well microtiter plate and cover or tape sealer
250-µl multichannel pipettor and appropriate tips
Microtiter plate mixer
Microtiter plate reader

1. Draw a template for placement of samples and standards on the microtiter plate.
2. Select a protein to use as the standard (see Strategic Planning). Prepare a dilution series with the same buffer used to dilute the samples to cover the range of 10 to 160 mg/ml.
3. In triplicate, add 5.0 µl of each diluted standard or sample into the appropriate microtiter plate wells. Use the buffer or diluent that was used to dilute the standard and samples for the blank wells.
4. Using a multichannel pipettor, add 250 µl biuret reagent to each well. Mix well on a plate shaker for 30 sec.
5. Cover the plate and incubate at room temperature for 10 min.
6. Mix again. Remove the plate cover and measure the color in each well of the plate at 540 nm (A_{540}) in a microtiter plate reader.
7. Prepare a standard curve by graphing the average net or blank-corrected A_{540} values for each standard versus its concentration in milligrams per milliliter.
8. Determine the sample concentration by interpolating from the plot (see Strategic Planning).

THE COOMASSIE DYE–BINDING (BRADFORD) ASSAY FOR DETERMINING TOTAL PROTEIN

BASIC PROTOCOL 4

The Coomassie dye–based protein-binding assays have the advantage of being the fastest and the easiest to perform (Fig. B1.1.7). In addition, the assay is performed at room temperature and no special equipment, other than a spectrophotometer, is required. Briefly, the sample is added to the ready-to-use reagent and, following a short incubation, the resultant blue color is measured at 595 nm versus deionized water.

In 1976, Marion Bradford introduced the first Coomassie dye–based reagent for the rapid colorimetric detection and quantitation of total protein. The Coomassie dye (Bradford) protein assay reagents have the advantage of being compatible with most salts, solvents, buffers, thiols, reducing substances, and metal chelating agents encountered in protein samples.

Materials

Sample buffer or solvent
Protein standard (e.g., 2 mg/ml BSA; see recipe)
Protein sample
Coomassie dye reagent (Pierce or Bio-Rad; also see recipe)

1. Prepare a dilution series from protein standard (e.g., 2 mg/ml BSA) and sample buffer to cover the range 100 to 1000 µg/ml.

   Bovine serum albumin (BSA) is often used as a calibration standard, but it has greater general dye-binding capacity than most proteins (Bradford, 1976).

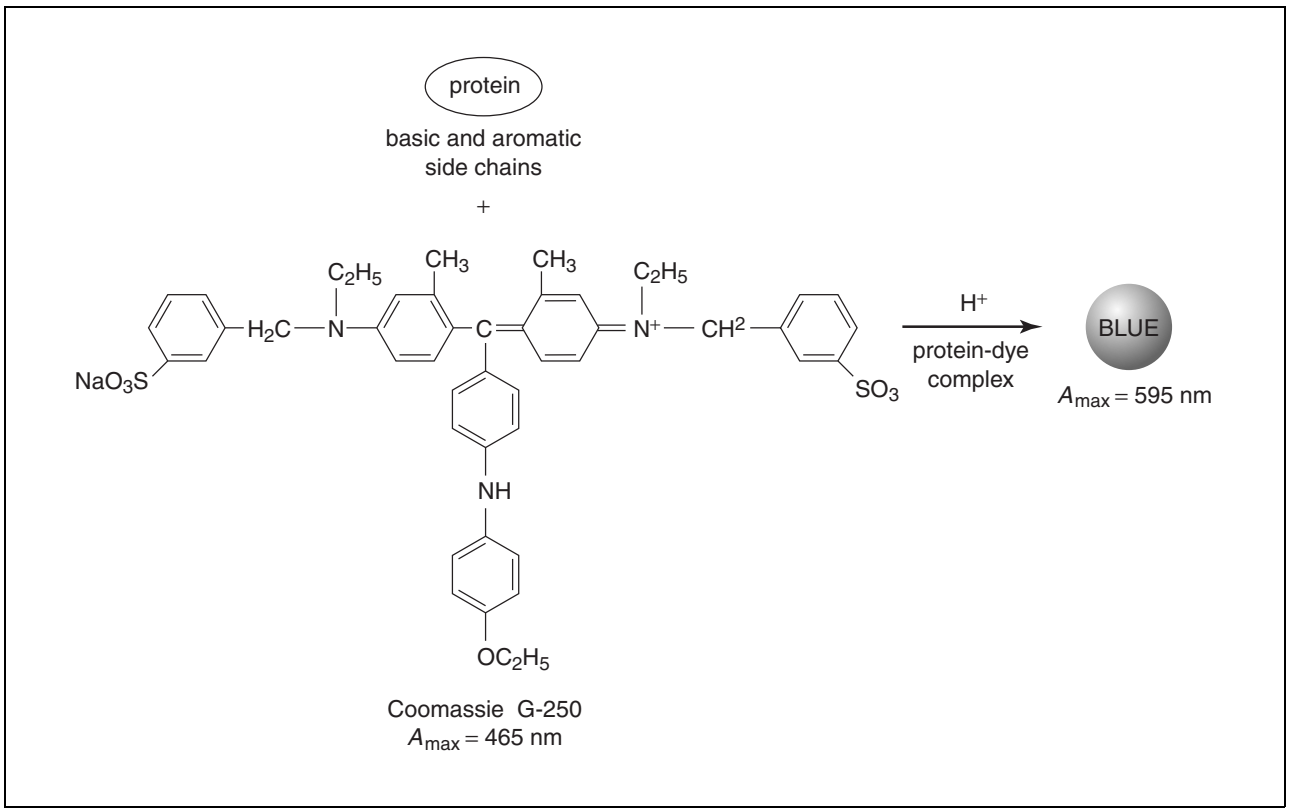

Figure B1.1.7 The reaction schematic for the Coomassie Protein Assay.

2. Dispense 0.1 ml standard, sample, or buffer to appropriately labeled tubes.

   If commercially prepared Coomassie reagent is used, follow the manufacturer's instructions. Commercial reagents tend to produce less turbidity and more consistent results.

3. Add 5 ml Coomassie dye reagent. Vortex immediately.
4. Incubate 10 min at room temperature.
5. Vortex each tube just before measuring the absorbance at 595 nm (A_{595}).
6. Plot a standard curve by graphing the average net or blank-corrected A_{595} values for the standard versus its protein concentration in micrograms per milliliter.
7. Determine the sample concentration by interpolation from the standard curve (see Strategic Planning).

ALTERNATE PROTOCOL 6

THE COOMASSIE PLUS PROTEIN ASSAY FOR DETERMINATION OF TOTAL PROTEIN

Several companies offer modified Bradford Coomassie dye–based protein assay reagents. Perhaps the most popular such reagent is the Protein Assay Reagent available from Bio-Rad. The Coomassie Plus Protein Assay Reagent available from Pierce is another modification of the Bradford formulation. In addition to the attributes cited above, the Coomassie Plus Protein Assay Reagent has the unique advantage of producing a linear response curve within a portion of its working range. For BSA, the response curve is linear from 125 to 1000 µg/ml and for bovine gamma globulin (BGG), the response curve is linear from 125 to 1500 µg/ml. The complete working range of the assay covers the concentration range from 100 to 2000 µg/ml for the tube protocol and from 1 to 25 µg/ml for the microtiter protocol (see Alternate Protocol 7).

Table B1.1.4 Troubleshooting Guide for Coomassie Plus Protein

Problem	Possible cause	Solution
Blank A_{595} is normal, but standards show less color than expected	Improper reagent storage	Store reagent refrigerated
	Reagent still cold	Warm to room temperature before use
	Color measured at the wrong wavelength	Measure the color at 595 nm
Blank and standards are normal, but samples show little color	Low molecular weight of sample protein (<3000 kDa)	Use the BCA (see Basic Protocol 2) or Lowry protein assay (see Basic Protocol 1)
A precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute the sample
		Precipitate the protein with TCA, dissolve pellet in 50 mM NaOH
All tubes (including the blank) are dark blue	Strong alkaline buffer or reagent raises reagent's pH	Dialyze or dilute the sample
	Sample volume too large, reagent pH raised	Maximum of 1 part sample and 1 part reagent
Need to read color at a different wavelength	Colorimeter does not have 595 nm filter	Color may be read at any wavelength between 575 nm and 615 nm

The main disadvantage of all Bradford-type protein assay reagents is that they are not compatible with surfactants at concentrations routinely used to solubilize membrane proteins. With some exceptions, the presence of a surfactant in the sample, even at low concentrations, causes precipitation of the reagent. Table B1.1.4 is a brief troubleshooting guide for this technique.

Additional Materials *(also see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay Reagent

1. Prepare a dilution series with 2 mg/ml BSA and buffer to cover a range from 100 to 2000 µg/ml.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock BSA standard that was used with the samples.

2. Allow the Coomassie Plus Protein Assay reagent to come to room temperature. Mix the assay reagent well by gentle inversion before use.
3. In duplicate, dispense 50 µl standard, sample, or diluent (blank) into appropriately labeled test tubes.
4. Add 1.5 ml Coomassie Plus Protein Assay Reagent to each tube. Mix each tube well by vortexing 2 to 3 sec.
5. Let the tubes stand 10 min at room temperature.
6. Mix each tube again just before measuring the absorbance at 595 nm (A_{595}).

   Disposable polystyrene cuvettes eliminate the job of cleaning dye-stained quartz or glass cuvettes.

7. Plot a standard curve by graphing the average net or blank-corrected A_{595} reading for each standard versus its concentration in micrograms per milliliter.

   Example color response curves for BSA and BGG are shown in Figure B1.1.8.

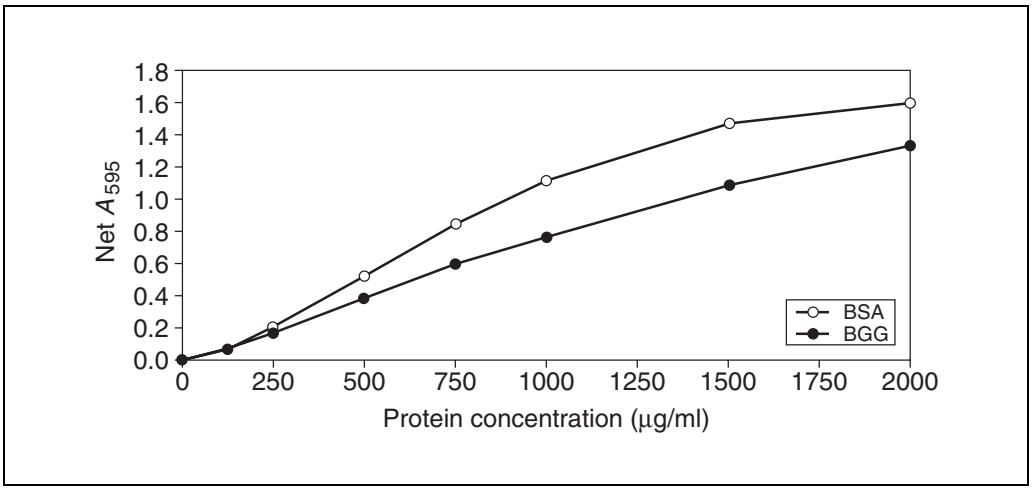

Figure B1.1.8 Graph of the color response curves obtained with Pierce's Coomassie Plus Protein Assay Reagent using bovine serum albumin (BSA) and bovine gamma globulin (BGG). The standard tube protocol was performed and the color was measured at 595 nm in a Hitachi U-2000 spectrophotometer.

8. Determine the protein concentration for each sample by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

ALTERNATE PROTOCOL 7

MICROTITER PLATE COOMASSIE ASSAY FOR TOTAL PROTEIN

This microtiter plate assay has a working range of 1 to 25 μg/ml.

Additional Materials *(see Basic Protocol 4)*

Coomassie Plus Protein Assay Reagent Kit (Pierce) containing Coomassie Plus Protein Assay reagent
96-well microtiter plate
300-μl multichannel pipettor
Microtiter plate mixer
Microtiter plate reader

1. Allow the Coomassie Plus Protein Assay Reagent to come to room temperature. Once the reagent is at room temperature, mix the reagent well by gentle inversion of the bottle.
2. Draw a template for placement of samples and standards on a 96-well microtiter plate.
3. In triplicate, dispense 10 μl of each diluted BSA standard, sample, or diluent (blank) into the appropriate wells of a 96-well microtiter plate.

   If possible, use the same diluent or buffer cocktail for the blanks and for diluting the stock standard that was used with the samples.

4. Dispense 300 μl Coomassie Plus Protein Assay reagent into each well with a multichannel pipettor. Mix the plate well on a plate shaker for 30 sec.
5. Let the plate incubate 10 min at room temperature.
6. Just before reading, mix the plate again, then measure the absorbance at 595 nm (A_{595}) on a plate reader.

7. Prepare a standard curve by graphing the average net or blank corrected A_{595} values for each standard versus its concentration in micrograms per milliliter.

8. Determine the sample concentration by interpolating from the standard curve (see Strategic Planning). Calculate the average total protein concentration for each sample from the replicates.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCA reagent A

1 g 4,4′-dicarboxy-2,2′-biquinoline, disodium salt (Na_2BCA; Pierce or Sigma; 1% w/v final)
2 g $Na_2CO_3 \cdot H_2O$ (2% w/v final)
160 mg sodium tartrate dihydrate (0.16% w/v final)
0.4 g NaOH (0.4% w/v final)
0.95 g $NaHCO_3$ (0.95% w/v final)

Dissolve all of the above chemicals except the sodium bicarbonate in deionized water and adjust the final volume to 100 ml. Adjust the pH to 11.25 by adding the sodium bicarbonate a little at a time. Store this alkaline reagent in a plastic container 1 to 3 weeks at room temperature, longer at 4°C.

Only the disodium salt of Na_2BCA is soluble at neutral pH; the free acid is not readily soluble.

BCA reagent B

4 g $CuSO_4 \cdot 5H_2O$ (4% w/v final)
100 ml H_2O
Store up to 6 months at room temperature

Biuret total protein reagent

0.6 mol/liter sodium hydroxide
12.0 mmol/liter copper sulfate
31.9 mmol/liter sodium potassium tartrate
30.1 mmol/liter potassium iodide
Store up to 6 months at room temperature

This reagent is based upon the candidate reference method for the determination of total protein in serum developed by Doumas et al. (1981). It is available from Sigma Diagnostics.

BSA, 2 mg/ml (w/v)

200 mg BSA (crystallized or lyophilized or one of the Cohn Fraction V preparations which are 96% to 98% protein and 3% to 4% water) in 100 ml of 0.9% saline containing 0.05% sodium azide. Store up to 6 months at 4°C.

Coomassie dye reagent

100 mg Coomassie Brilliant Blue G-250 (0.01% w/v final)
50 ml 95% ethanol (4.7% final)
100 ml 85% (w/v) phosphoric acid (8.5% w/v final)

In a small container, dissolve the dye in the ~25 ml ethanol, add the dye/ethanol solution to 800 ml of deionized water. Use the remaining ethanol to rinse the dye/ethanol container and add the rinses to the formulation. While mixing, slowly add the acid to the formulation and adjust the final volume to 1000 ml with deionized water. Filter the reagent through a single pad of Whatman no. 2 filter paper. Store up to 1 month at room temperature in a glass container.

continued

Coomassie Brilliant Blue G-250 (color index 42655) is available from a number of different suppliers (e.g., ACROS, Aldrich, AMRESCO, Bio-Rad, Fisher Biotech, Fluka, ICN Biomedicals, J.T. Baker, Research Organics, Serva, Sigma, or USB).

Lowry's reagent A

21.2 g sodium carbonate (2% w/v)
40 ml 1 N NaOH (or 4.0 g NaOH; 0.1 N final)
Add distilled or deionized water to 1 liter
Make fresh daily

Lowry's reagent B

0.5 g $CuSO_4 \cdot 5H_2O$ (0.5% final)
1 g sodium tartrate (1% final)
Make fresh daily

Sodium tartrate may be replaced with disodium tartrate, potassium sodium tartrate, or sodium citrate for better solubility.

Lowry's reagent C (1 N Folin phenol reagent)

Dilute 2 N Folin phenol (Sigma, Fisher, or VWR) with an equal volume water. Prepare immediately before use.

Lowry's reagent D (reagent A and B mix)

Mix 1 vol reagent B and 50 vol reagent A (see recipes). Prepare it immediately before use.

Lowry's reagent D′

Add 2 ml of 10% sodium dodecyl sulfate (SDS; *APPENDIX 2A* for 20% solution) in deionized water to each 100 ml Lowry's reagent D (see recipe). Prepare immediately before use.

COMMENTARY

Background Information

The modified Lowry protein assay

Although the exact mechanism of color formation reaction in the Lowry protein assay remains poorly understood, it is known that the color producing reaction with protein occurs in two distinct steps. As seen in Figure B1.1.1, protein is first reacted with alkaline cupric sulfate in the presence of tartrate during a 10-min incubation at room temperature. During this incubation, a tetradentate copper complex forms from four peptide bonds and one atom of copper. The tetradentate copper complex is light blue in color (this is the "biuret reaction"). Following the 10-min incubation, Folin phenol reagent is added. It is believed that color enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic/phosphotungstic acid complex (i.e., the Folin phenol reagent).

The reduced phosphomolybdic/phosphotungstic acid complex produced by this reaction is intensely blue in color. The Folin phenol reagent loses its reactivity almost immediately upon addition to the alkaline working reagent/sample solution. The blue color continues to intensify during a 30-min room temperature incubation. It has been suggested by Lowry et al. (1951) and by Legler et al. (1985) that during the 30-min incubation, a rearrangement of the initial unstable blue complex leads to the stable final blue colored complex, which has higher absorbance.

For small peptides, the amount of color increases with the size of the peptide. The presence of any of five amino acid residues (tyrosine, tryptophan, cysteine, histidine, and asparagine) in the peptide or protein backbone further enhance the amount of color produced, because they contribute additional reducing equivalents for further reduction of the phosphomolybdic/phosphotungstic acid complex. With the exception of tyrosine and tryptophan, free amino acids will not produce a colored product with the Lowry reagent; however, most dipeptides can be detected. In the absence of any of the five amino acids listed above, proteins containing proline residues have a lower

color response with the Lowry reagent because it interferes with complex formation.

The final blue color is optimally measured at 750 nm, but it can be measured at any wavelength between 650 nm and 750 nm with little loss of color intensity. Dr. Lowry (1951) recommended reading the color at 750 nm because few other substances absorb light at that wavelength. The amount of light absorbed at 750 nm is directly proportional to the amount of protein in the sample, but the color response curve produced is nonlinear.

The sensitivity of the Modified Lowry Protein Assay Reagent is greatly enhanced over that of the Biuret total protein reagent. The working range of the method covers the total protein range from 1 to 1500 μg/ml. In comparison, the working range for the Biuret assay is from 5 to 160 mg/ml.

The modified Lowry protein assay reagent will form precipitates in the presence of surfactants or potassium ions. The problem of precipitation that is caused by the presence of potassium ions in the sample can sometimes be overcome by centrifuging the tube and reading the color in the supernatant. Most surfactants will cause precipitation of the reagent even at very low concentrations. One exception is sodium dodecyl sulfate (SDS), which is compatible with the reagent at concentrations up to 1% (w/v) in the sample. Chelating agents interfere because they bind copper and thus prevent formation of the copper-peptide bond complex. Reducing agents and free thiols interfere, as they reduce the phosphotungstate-phosphomolybdate complex, immediately forming an intensely blue colored product upon their addition to the modified Lowry protein assay reagent.

The Coomassie plus protein assay

The primary advantage of the Coomassie plus protein assay is that it is generally compatible with most of the buffers and reagents found in samples and is unaffected by the presence of chelating agents, reducing agents, or free sulfhydryls in the sample.

The development of color in the Coomassie dye-binding methods has been associated with the presence of certain basic amino acids (primarily arginine, lysine, and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found on the protein. In the acidic environment of the Coomassie plus protein assay reagent, protein binds to the Coomassie dye. This results in a spectral shift of the reagent from the reddish/brown form of the dye, with an absorbance maximum at 465 nm, to the blue form of the dye, with an absorbance maximum at 610 nm (see Fig. B1.1.7).

The difference between the two forms is greatest at 595 nm; therefore, this is the optimal wavelength to measure the blue color from the Coomassie dye–protein complex. If desired, the blue color can be read at any wavelength between 575 and 615 nm. At the two extremes (575 and 615 nm) there is a loss of ~10% in the measured amount of color (absorbance) compared to the value obtained at 595 nm.

Free amino acids, peptides, and low molecular weight proteins do not produce color with the Coomassie plus protein assay reagent. In general the molecular weight of the peptide or protein must be at least 3000 kDa to be assayed with this reagent. In some applications this can be an advantage. The reagent has been used to measure "high molecular weight proteins" during fermentation in the beer brewing industry.

One disadvantage of any Coomassie dye–based protein assay is that surfactants in the sample will cause precipitation of the reagent. Another disadvantage is that the Coomassie plus protein assay reagent shows almost twice as much protein-to-protein variation as that obtained with the protein-copper chelation–based assay reagents (the Lowry protein assay reagent or the BCA protein assay reagent). While this is true, the reagent exhibits the least protein-to-protein variation of all Coomassie dye–based (Bradford) reagents. Since the Coomassie dye reagent is highly acidic, a small number of proteins cannot be assayed with this reagent due to poor solubility. In addition, the glass or quartz cuvettes routinely used to hold the solution in the spectrophotometer while the color intensity is being measured are stained by Coomassie dye–based reagents.

The BCA protein assay

The BCA protein assay is a modification of the Lowry protein assay in which enhanced color production is due to the reaction of reduced copper with bicinchoninic acid (BCA). This reagent has a unique advantage over the Lowry protein assay reagent and the Coomassie plus protein assay reagent because it is compatible with samples that contain up to 5% (v/v) surfactants (detergents). Unlike the Lowry protein assay, all reactants needed are present in the BCA working reagent. Large numbers of

tubes can be run without regard to the limits imposed in the Lowry assay by the need to add a second reagent at a precise time interval.

The working range of the BCA protein assay reagent is from 20 to 2000 µg/ml for both the standard tube and the standard microtiter plate protocols. Since the color reaction is not a true end-point reaction, this allows more protocol flexibility. By increasing the incubation temperature, the sensitivity of the reagent can be increased. When using the enhanced tube protocol (incubating at 60°C for 30 min), the working range for the assay shifts to 5 to 250 µg/ml and the minimum detection level becomes 5 µg/ml.

Although the mechanism of color formation with protein for the BCA Protein Assay Reagent is similar to that of the Lowry reagent, there are several significant differences. The BCA protein assay combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the so-called biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion (see Fig. B1.1.3). The resultant BCA/Cu^{+} complex is water-soluble and exhibits a strong linear absorbance maximum at 562 nm with increasing protein concentrations. If desired, the purple color may be measured at any wavelength between 550 nm and 570 nm with minimum (<10%) loss of signal.

The reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of any of four amino acid residues (tyrosine, tryptophan, cysteine, or cystine) in the amino acid sequence of the protein. Unlike the Coomassie dye–binding (Bradford) methods, which require a minimum mass of protein to be present for the dye to bind, the presence of only a single amino acid residue in the sample may result in the formation of a colored BCA-Cu^{+} chelate. This is true for any of the four amino acids cited above. Studies done with di- and tripeptides indicate that the total amount of color produced is greater than can be accounted for by the simple addition of the color produced with each BCA-reactive amino acid, so the peptide backbone must contribute to the reduction of copper as well.

The rate of color formation is dependent on the incubation temperature, the types of protein present in the sample and the relative amounts of reactive amino acids contained in the proteins. The recommended protocols do not result in end-point determinations, so the incubation periods were chosen to yield maximal color response in a reasonable time frame.

The protein-to-protein variation in the amount of color produced with the BCA Protein Assay Reagent (CV = 15% for the group of 14 proteins at 1000 µg/ml in the standard tube protocol) is similar to that observed for the modified Lowry protein assay reagent.

The biuret total protein assay

Riegler (1914) introduced the biuret reaction as a method for the estimation of albumin in urine in 1914. Modifications and improvements to the biuret method were made by Autenrieth and Mink (1915), Hiller (1926), and Fine (1935). All of these early methods required the separate addition of a sodium hydroxide solution and the separate addition of a copper sulfate solution to the sample. The methods suffered from poor precision due to ineffective mixing and variation in reaction time during mixing. The first "one solution" biuret reagent was introduced by Kingsley (1942) for use in measuring total protein, albumin, and globulin in human serum. Weichselbaum (1946) and Gornall et al. (1949) modified Kingsley's biuret reagent by decreasing the sodium hydroxide concentration to prevent the formation of precipitates and by adding sodium potassium tartrate to stabilize the reagent. Potassium iodide was added to prevent the autoreduction of Cu^{2+}. Goa (1953) published on the use of the biuret reagent for determining total protein in human cerebrospinal fluid.

Sigma Diagnostic's Biuret Total Protein Reagent is based upon the candidate reference method formulation used for the determination of total protein in serum developed by Doumas et al. (1981). Because the primary application for this reagent is the determination of total protein in serum, most of the studies regarding assay precision, assay linearity, and interfering substances have been done on clinical samples. On samples with total protein concentrations in the range of 26 to 121 mg/ml, within-run and between-run precision was found to be excellent (CV's ranged from 4.2% to 1.4%). Lipids, bilirubin, hemoglobin, dextran, and certain drugs have been shown to interfere with the total protein results obtained with the Biuret Total Protein Reagent. Interference caused by the presence of lipid in the sample is due to turbidity. Interference caused by the presence of bilirubin or hemoglobin is small, almost negligible. Dextran causes precipitation in the

reaction mixture during color development; however, centrifuging the reaction mixture before reading the color can minimize this. The Biuret Total Protein Reagent contains 0.6 mol/liter sodium hydroxide, 12.0 mmol/liter copper sulfate, 31.9 mmol/liter sodium potassium tartrate, and 30.1 mmol/liter potassium iodide. It should be stored at ambient room temperature until the expiration date shown on the label. Certain drugs and other substances are known to influence circulating levels of total protein (Young, 1990).

Critical Parameters

The modified Lowry protein assay reagent

The modified Lowry protein assay reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold modified Lowry protein assay reagent will result in low A_{750} values.

The protocol requires that the Folin phenol reagent be added to each tube precisely at the end of the 10-min incubation. At the alkaline pH of the Lowry reagent, the Folin phenol reagent is almost immediately inactivated; therefore, it is best to add the Folin phenol reagent at the precise time while simultaneously mixing each tube. Because it is somewhat cumbersome, it requires some practice to do the assay well. From a practical point of view, it also limits the total number of tubes that can be done in a single run. If a 10-sec interval between tubes is used, the maximum number of tubes that can be done within 10 min is 60 (10 sec/tube × 60 tubes = 600 sec or 10 min).

The Coomassie plus protein assay reagent

The Coomassie plus protein assay reagent must be refrigerated for long-term storage. If the entire bottle of reagent will be used within a reasonable time, the reagent may be stored at ambient room temperature (18° to 26°C) for up to 1 month. Reagent that has been left at room temperature for more than a month may show lower color response with protein, especially at the higher end of the working range. If the reagent has been stored refrigerated, it must be warmed to room temperature before use. The use of cold Coomassie plus protein assay reagent will result in low A_{595} values.

The Coomassie plus protein assay reagent must be mixed gently by inversion just before use. The Coomassie dye in the reagent spontaneously forms loosely associated dye-dye aggregates upon standing. These aggregates may become visible after the reagent has been standing for as little as 60 min. Gentle mixing of the reagent by inversion of the bottle will uniformly disperse the dye-dye aggregates. After binding to protein, the dye also forms protein-dye-protein-dye aggregates. Fortunately, these aggregates can be dispersed easily by mixing the reaction tube. This is common to all Coomassie dye–based (Bradford) protein assay reagents. Since these aggregates form relatively quickly, it is a good idea to routinely mix (vortex for 2 to 3 sec) each sample just before measuring the color.

Bradford-type reagents containing Coomassie dye will leave a blue stain on glass or quartz cuvettes. The stain can be removed by washing the cuvettes in a dilute detergent solution in hot tap water, rinsing with water, then washing with methanol or ethanol, and finally rinsing with deionized water. Disposable plastic (polystyrene) cuvettes are strongly recommended because they eliminate the need to clean; however, these cuvettes are not compatible with samples containing organic solvents (e.g., acetone, DMF, acetonitrile).

The BCA protein assay reagent

Since the BCA protein assay is not a true end-point assay, the amount of color produced varies with the incubation time and the incubation temperature. While this allows considerable flexibility in optimizing the BCA assay for each application, it also requires that the optimized procedure be followed exactly every time the assay is done.

At room temperature, following an initial lag phase, the rate of color formation remains relatively constant for hours. If the incubation time alone is increased, the total amount of color produced by a given mass of protein increases.

If the incubation temperature is increased, the rate of color formation increases and the total amount of color produced by a given mass of protein increases. After cooling the reaction mixture back to room temperature, the rate of color development slows from its initial rate. As both time and temperature are increased, the total amount of color produced by a given mass of protein approaches a maximum. This is ap-

parent from the dramatic decrease in the rate of color formation upon cooling to room temperature following incubation at 60°C for 30 min.

Above 75°C, a black precipitant forms in the BCA reaction mixture and the absorbance at 562 nm of the blank increases dramatically. This appears to be caused by the formation of copper oxide at high temperature.

For greatest accuracy and precision when comparing sets of data from multiple runs, a set of standards must be included with each run, and the standards and the samples must be treated exactly the same.

The biuret total protein reagent

The biuret total protein reagent is considerably less sensitive to total protein than the other three protein assay reagents discussed in this unit. This limits the applications in which the Biuret reagent can be used. Since the primary use of the Biuret reagent has been for serum total protein in the clinical laboratory, there is little published information about its compatibility with substances and reagents common to nonclinical samples.

Anticipated Results

Standard curves

Typical standard curves are shown in Figures B1.1.2, B1.1.4, B1.1.6, and B1.1.8 for each of the four assay methods. In each case, the tube protocols were performed in duplicate on diluted BSA or BGG standard. The color in each tube was measured at the appropriate wavelength in a dual-beam spectrophotometer. The net absorbance for each sample was plotted versus its protein concentration.

Figure B1.1.2 shows the color response curves obtained with the Modified Lowry Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 750 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure B1.1.4 shows the color response curves obtained with the BCA Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 562 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the response curve for BGG is higher than the response curve for BSA.

Figure B1.1.6 shows the color response curves obtained with the Biuret total protein reagent using BSA and BGG. The graph shows the net absorbance at 540 nm versus the protein at eight concentrations from 5 to 100 mg/ml.

Figure B1.1.8 shows the color response curves obtained with the Coomassie Plus Protein Assay Reagent using BSA and BGG. The graph shows the net absorbance at 595 nm versus the protein concentration at seven concentrations from 125 to 2000 µg/ml for each protein. Note that the color response curve for BGG is lower than the response curve for BSA.

Protein-to-protein variation

Each protein in a sample is unique and can demonstrate that individuality in protein assays as variation in the color response. Such protein-to-protein variation refers to differences in the amount of color (absorbance) that are obtained when the same mass (microgram or milligram) of various proteins are assayed concurrently (i.e., in the same run) by the same method. These differences in color response relate to differences among proteins due to amino acid sequence, isoelectric point (pI), secondary structure, and the presence of certain side chains or prosthetic groups.

To analyze protein-to-protein variation for each method, a group of fourteen proteins was assayed in duplicate using the standard tube protocol in a single run. The net (blank corrected) average absorbance for each protein was calculated. To make it easier to interpret, the net absorbance for each protein was expressed as a ratio to the net absorbance for BSA. If a protein has a ratio of 0.80, it means that the protein produces ~80% of the color that is obtained for an equivalent mass of BSA.

Table B1.1.5 demonstrates the relative degree of protein-to-protein variation that can be expected with the different protein assay methods. This differential may be a consideration in selecting a protein assay method, especially if the relative color response ratio of the protein in the samples is unknown. As expected, the protein assay methods that share the same basic chemistry show similar protein-to-protein variation.

The protein-to-protein variation observed with the various protein assay methods makes it obvious why the largest source of error for protein assays is the choice of protein for the standard curve. If the sample contained IgG as the major protein and BSA was used for the standard curve, the estimated total protein concentration of the sample will be inaccurate. Whether the concentration was underestimated or overestimated depends upon which total protein assay method was used. If the Coomassie

Table B1.1.5 Protein-to-Protein Variation[a]

Protein tested	Ratios obtained with the BCA method	Ratios obtained with the Coomassie plus method	Ratios obtained with the modified Lowry method
Albumin, bovine (BSA)	1.00	1.00	1.00
Aldolase, rabbit	0.85	0.74	0.94
α-Chymotrypsinogen, bovine	1.14	0.52	1.17
Cytochrome C, horse	0.83	1.03	0.94
Gamma globulin, bovine	1.11	0.58	1.14
IgG, bovine	1.21	0.63	1.29
IgG, human	1.09	0.66	1.13
IgG, mouse	1.18	0.62	1.20
IgG, rabbit	1.12	0.43	1.19
IgG, sheep	1.17	0.57	1.28
Insulin, bovine pancreas	1.08	0.67	1.12
Myoglobin	0.74	1.15	0.90
Ovalbumin	0.93	0.68	1.02
Transferrin, human	0.89	0.90	0.92
Average ratio	1.02	0.73	1.09
Standard Deviation (SD)	0.15	0.12	0.13
Coefficient of Variation	0.15	0.29	0.12

[a]The protein-to-protein variation in color response was measured at 1000 µg/ml for each protein in duplicate using the standard tube protocol. Within each assay, the average net or blank corrected absorbance was determined for each protein. The average net absorbance for each protein was divided by the average net absorbance obtained with BSA and expressed as a ratio. The standard deviation (SD) and the coefficient of variation (CV) is presented for the fourteen proteins assayed on the three methods. By comparing the CV's, the relative degree of protein-to-protein variation to be expected with the three methods can be assessed.

Plus Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be underestimated by ~40%. (From Table B1.1.5, the response ratio for IgG is ~0.58 for IgG compared to 1.00 for BSA.) If the BCA Protein Assay Reagent was used, the total protein (IgG) concentration in the sample would be overestimated by ~15%. (From Table B1.1.5, the response ratio for IgG is ~1.15 for IgG compared to 1.00 for BSA.) On the other hand, if BGG had been used for both standard curves, the total protein estimates for the sample would have been in much greater agreement between the two methods.

While Table B1.1.5 is useful because it provides an estimate of the protein-to-protein variation in color response that can be expected with each method, it does not tell the whole story. Because the comparisons were done at a single protein concentration, it is not apparent that the color response ratio also varies with changes in protein concentration.

Compatible and incompatible substances

An extensive list of substances that have been found to be compatible with each of the reagents is shown in Table B1.1.6. Each substance was assayed in duplicate using the standard tube protocol for each reagent. In addition to adding the substance to a sample containing 1000 µg/ml BSA, a blank sample containing only the substance was tested. When added to the sample, a substance was deemed to be compatible with a reagent if the blank-corrected absorbance for the sample containing the substance was within 10% of the blank-corrected absorbance for the sample containing only BSA (also at 1000 µg/ml).

Time Considerations

The amount of time required to complete a total protein assay will vary for the four colorimetric total protein assay methods described. For the purpose of providing an estimate of the amount of time required to perform a run by each method, it was assumed that the run included twenty samples and eight standards (including the blank) and that each sample or standard was assayed in duplicate using the standard tube protocol. The estimates do not include the time spent obtaining the samples or the time it takes to prepare the samples for

Table B1.1.6 Maximum Compatible Sample Concentration of 92 Substances[a]

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Detergents			
Brij 35	5.0%	0.062%	0.031%
Brij 56	1.0%	0.031%	0.062%
Brij 58	1.0%	0.016%	0.062%
CHAPS	5.0%	5.0%	0.062%
CHAPSO	5.0%	5.0%	0.031%
Deoxycholic acid	5.0%	0.04%	Not tested
Lubrol PX	1.0%	0.031%	0.031%
Nonidet P-40	5.0%	0.5%	0.016%
Octyl glucoside	5.0%	0.5%	0.031%
Octyl β-thioglucoside	5.0%	3.0%	Not tested
SDS (lauryl)	5.0%	0.016%	1.0%
SPAN 20	1.0%	0.5%	0.25%
Triton X-100	5.0%	0.062%	0.031%
Triton X-114	1.0%	0.062%	0.031%
Triton X-305	1.0%	0.125%	0.031%
Triton X-405	1.0%	0.25%	0.031%
Tween 20	5.0%	0.031%	0.062%
Tween 60	5.0%	0.025%	Not tested
Tween 80	5.0%	0.016%	0.031%
Zwittergent 3-14	1.0%	0.025%	Not tested
Salts and buffers			
ACES, pH 7.8	25 mM	100 mM	Not tested
Ammonium sulfate	1.5 M	1 M	Not compatible
Asparagine	1 mM	10 mM	5 mM
Bicine, pH 8.4	20 mM	100 mM	Not tested
Bis-Tris, pH 6.5	33 mM	100 mM	Not tested
Borate (50 mM), pH 8.5 (BupH pack)	Undiluted	Undiluted	Not tested
B-PER cell lysis reagent	Undiluted	Diluted 1:2	Not tested
Calcium chloride in TBS	10 mM	10 mM	Not tested
Carbonate/bicarbonate, Na (0.2 M), pH 9.4	Undiluted	Undiluted	Not tested
Cesium bicarbonate	100 mM	100 mM	50 mM
CHES, pH 9.0	100 mM	100 mM	Not tested
Cobalt chloride in TBS	0.8 mM	10 mM	Not tested
EPPS, pH 8.0	100 mM	100 mM	Not tested
Ferric chloride in TBS	10 mM	10 mM	Not tested
Glycine	1 mM	100 mM	100 mM
HEPES	100 mM	100 mM	1 mM
Imidazole, pH 10.2	50 mM	200 mM	25 mM
MES, pH 6.1	100 mM	100 mM	100 mM
0.1 M MES/0.9% NaCl, pH 4.7	Undiluted	Undiluted	Not tested

continued

Table B1.1.6 Maximum Compatible Sample Concentration of 92 Substances[a], *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
MOPS, pH 7.2	100 mM	100 mM	Not tested
Modified Dulbecco's PBS	Undiluted	Undiluted	Not tested
Nickel chloride in TBS	10 mM	10 mM	Not tested
Phosphate buffered saline (PBS), pH 7.2	Undiluted	Undiluted	Not tested
PIPES, pH 6.8	100 mM	100 mM	Not tested
RIPA lysis buffer, pH 8.0	Undiluted	Diluted 1:40	Not tested
Sodium acetate	200 mM	180 mM	200 mM
Sodium azide	0.2%	0.5%	0.2%
Sodium bicarbonate	100 mM	100 mM	100 mM
Sodium chloride	1.0 M	1 M	1 M
Sodium citrate, pH 4.8	200 mM	200 mM	Not tested
Sodium phosphate	100 mM	100 mM	100 mM
Tricine, pH 8.0	25 mM	100 mM	Not tested
Triethanolamine, pH 7.8	25 mM	100 mM	Not tested
Tris	250 mM	2 M	10 mM
TBS, pH 7.6	Undiluted	Undiluted	Not tested
25 mM Tris/192 mM glycine, pH 8.0	Diluted 1:3	Undiluted	Not tested
25 mM Tris/192 mM glycine/0.1% SDS, pH 8.0	Undiluted	Diluted 1:4	Not tested
Zinc chloride in TBS	10 mM	10 mM	Not tested
Reducing agents			
N-acetylglucosamine in PBS	10 mM	100 mM	Not tested
Ascorbic acid	Not compatible	50 mM	1 mM
Catecholamines	Not compatible	Not tested	Not tested
Creatinine	Not compatible	Not tested	Not tested
Glucose	10 mM	1 M	0.1 mM
Melibiose	Not compatible		
Potassium thiocyanate	3 M		
Thiol-containing agents			
Cysteine	Not compatible	10 mM	1 mM
Dithioerythritol (DTE)	1 mM	1 mM	Not compatible
Dithiothreitol (DTT)	1 mM	5 mM	Not compatible
2-Mercaptoethanol	0.01%	1 M	1 mM
Thimerosal	0.01%	0.01%	0.01%
Chelating agents			
EDTA	10 mM	100 mM	1 mM
EGTA	Not compatible	2 mM	1 mM
Sodium citrate, pH 4.8	200 mM	200 mM	0.1 mM
Solvents/miscellaneous			
Acetone	10%	10%	10%
Acetonitrile	10%	10%	10%

continued

Table B1.1.6 Maximum Compatible Sample Concentration of 92 Substances[a], *continued*

Substance tested	BCA method	Coomassie plus method	Modified Lowry method
Aprotinin	10 mg/liter	10 mg/liter	10 mg/liter
DMF	10%	10%	10%
DMSO	10%	10%	10%
Ethanol	10%	10%	10%
Glycerol (fresh)	10%	10%	10%
Guanidine·HCl	4 M	3.5 M	100 mM
Hydrochloric acid	100 mM	100 mM	100 mM
Leupeptin	10 mg/liter	10 mg/liter	10 mg/liter
Methanol	10%	10%	10%
Phenol Red	Not compatible	0.5 mg/liter	0.1 mg/liter
PMSF	1 mM	1 mM	1 mM
Sodium hydroxide	100 mM	100 mM	100 mM
Sucrose	40%	10%	7.5%
TLCK	0.1 mg/liter	0.1 mg/liter	0.01 mg/liter
TPCK	0.1 mg/liter	0.1 mg/liter	0.1 mg/liter
Urea	3 M	3 M	3 M
o-vanadate in PBS	1 mM	1 mM	Not tested

[a]Taken from the Protein Assay Technical Handbook, Pierce Chemical, 1999.

Table B1.1.7 Estimated Time Requirements

Method	Incubation time(s)	Estimated total assay time
Modified Lowry Reagent	10 and 30 min	110 min (1 hr, 50 min)
Coomassie Plus Reagent	10 min	80 min (1 hr, 20 min)
BCA Reagent	30 min	100 min (1 hr, 40 min)
Biuret Reagent	10 min	80 min (1 hr, 20 min)

analysis, but they do include the incubation time(s) plus an estimate of the time it takes to do the following:

1. Prepare (dilute) the standard protein in the diluent buffer (10 min).
2. Organize the run and label the tubes (5 min).
3. Pipet the samples and reagents into the tubes (10 min).
4. Mix or incubate the tubes or plates (varies).
5. Measure the color produced in the tubes (15 min).
6. Graph the standard curve, calculate, record, and report the results (30 min).

For each of the four methods, a run of 20 samples (unknowns) and the standard curve (each done in duplicate) can be completed in the time estimated in Table B1.1.7.

Acknowledgements

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Beyer, R.E. 1983. A rapid biuret assay for protein of whole fatty tissues. *Anal. Biochem.* 129:483-485.

Accurate total protein quantitation of fatty tissues was achieved by adding 0.1 ml 10% sodium deoxycholate, pH 8.0 and 2.9 ml biuret reagent to each protein pellet following an acetone/ether wash step. After sonication, each sample was heated for 30 sec in a boiling water bath to develop full color.

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Found that the anionic form of the Coomassie dye reacts primarily with arginine residues within the macromolecular protein. Coomassie dye reacts to a lesser extent with other basic amino acid residues (His, Lys) and aromatic residues (Try, Tyr, Phe) present in macromolecular proteins, but not with the free amino acids. Dye binding is attributed to van der Waals forces and hydrophobic interactions. The interference seen with bases, detergents, and other compounds can be explained by their effects upon the equilibrium between the three dye forms (cationic, neutral, anionic).

Crowley, L.V. 1969. Interference with certain chemical analyses caused by dextran. *Am. J. Clin. Pathol.* 51:425.

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Sorenson, K. and Brodbeck, U. 1986. A sensitive protein assay method using micro-titer plates. *Experientia* 42:161-162.

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Tal, M., Silberstein, A., and Nusser, D. 1980. Why does Coomassie Brilliant Blue interact differently with different proteins? *J. Biol. Chem.* 260:9976-9980.

Analysis of Scatchard plots showed that the number of Coomassie dye ligands bound to each protein is

approximately proportional to the number of positive charges on the protein. About 1.5 to 3 dye molecules are bound to each positive charge on the protein.

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A modified biuret reagent was formulated (sodium tartrate replaces sodium potassium tartrate, the sodium hydroxide concentration is reduced, and potassium iodide was deleted). When the modified biuret reagent was mixed with samples containing 2% detergent (SDS or sodium cholate or Triton X-100), it resulted in less protein-to-protein variation among six proteins.

Weichselbaum, 1946. See above.

Used sodium potassium tartrate as a stabilizer and added potassium iodide to prevent autoreduction of the biuret reagent; however, this reagent was found to be unstable after long storage.

Wiechelman, K., Braun, R., and Fitzpatrick, J. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175:231-237.

Cysteine, cystine, tryptophan, tyrosine, and the peptide bond are capable of reducing Cu^{2+} to Cu^{+}, but the extent of color formation is not simply the sum of the contributions from the various color producing functional groups. At 60°C, tryptophan, tyrosine, and the peptide bond are more completely oxidized than they are at 37°C, which is observed by the much greater extent of color developed at the higher temperature.

Internet Resources

http://www.piercenet.com

This site allows access to Pierce's on-line product catalog and special notice of all new products and other Pierce publications. If specific information on a product is needed, the instruction booklet can be downloaded. If additional help is needed, the technical assistance department can be reached by e-mail.

http://www.sigma-aldrich.com

This site allows access to the Sigma Diagnostics product line and applications or other technical information on their products. Questions can be sent by e-mail to the technical assistance department.

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Determination of Total Nitrogen

Protein content of organic matters can be determined by two ways: (1) directly by using certain specific chemical or physical properties unique to proteins, or (2) indirectly by determining their nitrogen content. However, nitrogen determination is the most commonly used procedure for a protein assay and it has been the basis for various official and conventional methods currently in use for expressing total protein content of organic matters. The most commonly used nitrogen determination methods include various versions of Kjeldahl (AOAC, 1999a), Dumas (AOAC, 1999b), and combustion (AOAC, 1999c) methods. Although the Kjeldahl method has been most widely used for determination of nitrogen, Dumas and combustion methods offer the advantages of shorter analysis time and no requirement of hazardous chemicals. However, the inherent limitations of these three procedures must be recognized—the results are affected by nonprotein nitrogen.

In general, it is assumed that a mixture of pure proteins will contain 16% nitrogen. Thus, the protein content of a sample is calculated from the determined nitrogen content by multiplying by a nitrogen-to-protein conversion factor, 6.25 (i.e., 100/16). This general conversion factor is used for most foods because their nonprotein nitrogen content is negligible. However, a specific conversion factor can also be used when the correct value is known for a given commodity and its products, i.e., 5.70 for wheat, 6.38 for milk, and others (see Table B1.2.1).

NITROGEN DETERMINATION USING THE KJELDAHL METHOD

BASIC PROTOCOL 1

Since late in the 19th century, the classical Kjeldahl method has been recognized and accepted universally as the authoritative method of analysis for determining the protein content in a wide variety of ingredients and finished products. As a result of technical innovations there are currently available and in use semiautomated or fully automated protein analysis systems that are based on the classical Kjeldahl procedure.

The Kjeldahl method determines total nitrogen content and protein as the nitrogen content of the sample multiplied by a conversion factor. The sample is digested in sulfuric acid, using $CuSO_4/TiO_2$ as catalysts, converting N to NH_3, which is distilled and titrated (AOAC, 1999a). This method is applicable to a wide range of organic matters including raw materials, ingredients, and finished products from animals, cereals, and oilseeds.

Table B1.2.1 Conversion Factors from Percent Nitrogen to Percent Total Protein for Various Commodities and Their Products

Products	Conversion factors
Animal	6.25
Cottonseeds	5.30
Peanuts	5.46
Soybeans	5.71
Sunflower seeds	5.30
Safflower seeds	5.30
Coconut meat	5.30
Sesame seeds	5.30
Corn	6.25
Millet	5.83
Rice	5.95
Wheat	5.83

Contributed by Khee C. Rhee

Current Protocols in Food Analytical Chemistry (2001) B1.2.1-B1.2.9

Materials

Sample
Catalyst mixture (see recipe)
Alundum granules (Thomas Scientific)
Concentrated sulfuric acid (H_2SO_4; 95% to 98%, nitrogen-free)
Lysine·HCl, high purity (Sigma)
0.5 N hydrochloric or sulfuric acid standard solution (accurately standardized; see recipe for Standard Solutions)
Methyl red indicator solution (see recipe)
Concentrated NaOH solution (see recipe)
0.1 N NaOH standard solution (accurately standardized; see recipe for Standard Solutions)

Laboratory mill
500- to 900-ml Kjeldahl digestion flasks
8 to 14 mesh alumina boiling stones (Thomas Scientific)
500-ml or equivalent size titration beaker
Distillation apparatus: 500- to 900-ml Kjeldahl digestion flask connected to distillation trap by rubber stopper; distillation trap connected to condenser with low-S tubing (outlet of condenser tube should be <4 mm in diameter)

1. Grind sample through a suitable laboratory mill to uniform fineness of ~20 to 30 mesh.
2. Weigh 0.250 to 1.000 g sample into a 500- to 900-ml Kjeldahl digestion flask. Add the catalyst mixture, 0.5 to 1.0 g alundum granules and 20 ml of concentrated H_2SO_4.

   Add additional 1.0 ml H_2SO_4 for each 0.1 g fat or 0.2 g other organic matter if sample weighs >1 g.
3. Include at least one sample of an equivalent amount of nitrogen as the sample to be measured of high-purity lysine·HCl in each run to check correctness of digestion parameters.

   Also check entire method by analyzing NIST Standard Reference material no. 194 ($NH_4H_2PO_4$, certified 12.15% N).

   If recovery is not complete, make appropriate adjustments.
4. First, determine the heat input needed to bring 250 ml of water at 25°C to rolling boil in 5 min. Add a few boiling stones to the digestion flask to prevent overheating. Then heat samples in water under the predetermined conditions for a 5-min boil rate until dense white fumes clear the bulb of the flask (i.e., ~10 min). Swirl gently, and continue heating for an additional 40 min.

   IMPORTANT NOTE: *Reagent proportions, heat input, and digestion time are critical factors; do not change.*
5. Cool, cautiously add about 250 ml of water, and cool ~15 to 20 min to room temperature.

   IMPORTANT NOTE: *Add water as soon as possible to reduce amount of caking. If excessive bumping occurs during distillation, increase dilution water from 250 ml to ~300 ml.*
6. Prepare 500-ml titration beaker by adding appropriate volume of 0.5 N hydrochloric or sulfuric acid standard solution to a sufficient volume of water such that the condenser tip will be sufficiently immersed to trap all NH_3 evolved. Add 3 to 4 drops of methyl red indicator solution.

   In a Kjeldahl analysis, neither the volume nor the concentration of the acid solution used as the trap matter, so long as there are more equivalents of acid than of evolved ammonia

and the liquid level is high enough for the ammonia to be efficiently trapped. The object is to back-titrate all of the acid that has not been neutralized by the ammonia after the distillation. The actual volume and concentration used should be recorded, however, for use in the calculation.

7. Add additional 0.5 to 1.0 g alundum granules to cooled digestion flask. Add a sufficient volume of concentrated NaOH solution, slowly down the side of the flask. More than 45 ml of 45% NaOH is recommended to neutralize 20 ml of concentrated H_2SO_4 standard, and to render the mixture strongly alkaline. Immediately connect digestion flask to the distillation apparatus, mix completely, and distill at a 7.5-min boil rate until >150 ml distillate is collected in titration beaker.

   Optionally, 2 to 3 drops of tributyl citrate may also be added to reduce foaming.

8. Titrate excess standard acid in distillate with 0.1 N NaOH standard solution. Correct for blank determination on reagents.

9. Calculate nitrogen:

   $$\%N = \{[(N_{acid})(ml_{acid}) - (ml_{bk})(N_{NaOH}) - (ml_{NaOH})(N_{NaOH})] \times 1400.67\}/\text{mg sample}$$

   where ml_{NaOH} = milliliters of standard base needed to titrate sample; ml_{acid} = milliliters of standard acid used for that sample; ml_{bk} = milliliters of standard base needed to titrate 1 ml standard acid minus milliliters of standard base needed to titrate reagent blank carried through method and distilled into 1 ml standard acid; N_{acid} = normality of standard acid; N_{NaOH} = normality of standard base.

10. Calculate % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

NITROGEN DETERMINATION USING THE DUMAS METHOD

ALTERNATE PROTOCOL 1

Nitrogen, freed by pyrolysis and subsequent combustion, is swept by a carbon dioxide carrier into a nitrometer. The carbon dioxide is absorbed in KOH and the volume of residual nitrogen is measured and converted to equivalent protein by a numerical factor (AOAC, 1999b).

Materials

Samples
CuO-Pt catalyst (CuO wire form with 2.5% Pt reforming catalyst)
Co_3O_4
CuO fines
45% (w/v) KOH

Laboratory mill
No. 30 sieve
Bottles with caps
Nitrogen analyzer, consisting of combustion and collection and measuring systems (e.g., PE Biosystems)
Vycor combustion tubes
Stainless steel screen
Glass wool
11-mm glass rod
Aluminum (Al) combustion boats
Barometer, Hg type, readable to 0.1 mm

Prepare combustion tube

1. Grind samples in a laboratory mill to pass no. 30 sieve. Store in capped bottles.

2. Operate nitrogen analyzer in accordance with manufacturer's instructions.

3. After combustion furnaces have come to thermal equilibrium, turn combustion cycle control to START and let it proceed normally through the cycle. Observe indicated temperature on pyrometer of both upper and lower combustion furnaces at the end of the combustion portion of cycle.

   Furnace temperatures should be 850°C to 900°C; if not, adjust the temperatures.

4. Prepare combustion tube by inserting stainless steel screen in lower end of combustion tube (end farthest from trademark). In upper end, place enough glass wool to form a 6-mm plug when packed. With 11-mm glass rod, drive glass wool down to stainless steel plug.

5. Holding tube vertically, pour CuO-Pt catalyst directly from dispenser bottle into combustion tube until it reaches upper end of the trademark. Tap or vibrate tube on bench until reagent settles to approximate center of trademark.

6. Weigh and record empty Al combustion boat. Place sample in boat. Weigh and record weight of sample and combustion boat. Calculate the sample weight by taking the difference between weights. Weigh sample to nearest 0.01 mg. Use the following sample weights (mg) as guides to suitable sample sizes:

   Bermuda grass: 150 to 300 mg
   Rice bran, wheat shorts, and dehydrated alfalfa: 150 to 250 mg
   Range feed: 100 to 200 mg
   Cottonseed meal: 75 to 150 mg
   Edible soy protein: 50 to 150 mg

   To avoid weight changes, record weight within 1 min after sample and boat are placed on balance. If this is impossible, weigh sample inside a weighing bottle.

7. Turn combustion tube to horizontal, and carefully insert loaded sample boat into open end of tube. Slide or push boat, without spilling contents, until it reaches the trademark. Raise open end until tube forms a 60° to 70° angle to the horizontal plane. Tap or vibrate combustion tube on bench top while rotating tube between thumb and forefinger. Raise open end of tube and add 1 vol of Co_3O_4 and 1 vol of CuO fines equal to volume of sample.

   For a convenient means of adding above reagents to samples, place CuO fines and Co_3O_4 in a new combustion boat; add contents of boat, but not boat itself, to combustion tube; and rotate partially filled combustion tube between thumb and forefinger while varying angle of tube 20° to 45°. Continue rotating, tapping, and vibrating until sample is dislodged from boat and is thoroughly mixed with oxidizing agents.

8. Raise open end until tube forms a 60° to 70° angle; add additional CuO-Pt catalyst ~12 mm above the sample boat. Tap or vibrate gently to eliminate voids. Add CuO-Pt catalyst to within 20-mm top of tube, again tapping or vibrating gently to eliminate voids.

Run analyzer

9. Install prepared combustion tube in N_2 analyzer. Adjust 45% KOH solution meniscus to calibrating mark in nitrometer with digital readout meter. Record counter reading, R_1. Record syringe temperature, t_1, indicated on special scale thermometer.

   Counter reading should preferably lie between 500 and 1000 µl at this point. Vent control may be used to assist in arriving at this counter setting, if necessary.

10. Add 2 min more to combustion portion of cycle by turning auxiliary timer to setting 3. Turn combustion cycle control to START. Let analyzer proceed through its cycle. After cycle is complete and combustion cycle control has entered STAND-BY

section, readjust KOH meniscus to calibration mark with digital readout counter. Record new counter reading, R_2, and syringe temperature, t_2. Determine blank for instrument under same conditions as actual analysis except omit sample.

Once this is done, additional 2 min will be automatically programmed into each subsequent cycle.

11. Calculate total nitrogen.

    a. Record observed N_2 volume, $V_o = R_2 - R_1$, where V_o = observed N volume (µl), R_1 = initial counter reading, and R_2 = final counter reading.

    b. Determine corrected N_2 volume (in µl), $V_c = V_o - (V_b + V_t)$, where V_b = volume blank (µl), V_t = volume correction for temperature (µl) = $C_f(t_2 - t_1)$. C_f is obtained from Table B1.2.2 (based on final counter reading); t_1 and t_2 are initial and final temperatures in °K.

    c. Determine corrected barometric pressure, $P_c = P_o - (P_b + P_v)$, where P_o = observed barometric pressure (mm Hg), P_b = barometric temperature correction (from Table B1.2.3), and P_v = pressure correction for vapor pressure of KOH solution (from Table B.1.2.4).

Table B1.2.2 Volume Correction for Temperature Correction Factor (C_f) (µl/°K)[a]

Final counter reading (µl)	C_f (for nitrometers with check values below)
0	12
5,000	29
10,000	45
15,000	62
20,000	79
25,000	95
30,000	112
35,000	129
40,000	145
45,000	162
50,000	179

[a]Volume correction, $V_f = C_f(t_2 - t_1)$

Table B1.2.3 Barometric Temperature Correction (P_b)

Temperature (°C)	Correction (P_b)	
	For 700-749 mm Hg	For 750-780 mm Hg
10	1.2	1.3
15	1.8	1.9
20	2.3	2.5
25	2.9	3.1
30	3.5	3.7
35	4.1	4.3

Table B1.2.4 Pressure Correction (P_v) for Vapor Pressure of KOH[a,b]

Temperature (°K)	P_v (mm Hg)
288	4.1
293	5.7
298	7.4
303	9.6
308	12.5
313	16.5

[a]For practical purposes, temperature of KOH is same as syringe.
[b]Note that: empirical approximation of ($P_b + P_v$) = 11.0 will be satisfactorily accurate for P_o between 740 and 780 mm Hg and syringe temperature between 298° and 305°K.

d. Calculate %N = $(P_c \times V_c \times 0.0449)/(T \times W)$, where T = final syringe temperature in °K and W = sample weight in milligrams.

Example:
P_o = 750.1 mm Hg at 25°C; W = 148.91 mg
Counter readings—blank: 500 µl (start); 524 µl (finish)
Counter readings—sample: 5246 µl (start); 955 µl (finish)
t_1 = 302.7°K, t_2 = 303.0°K, V_o = 6955 − 524 = 6431 µl
$V_c = 6431 - [24 + C_f(t_2 - t_1)] = 6431(24 + 35 \times 0.3) = 6396$ µl
$P_c = 750.1 - (3.1 \times 9.6) = 737.4$
$\%N = (737.4 \times 6396 \times 0.04493)/(303.0 \times 148.91) = 4.69$

e. Calculate % protein = %N × 6.25, or %N × 5.70 in case of wheat grains.

ALTERNATE PROTOCOL 2

NITROGEN DETERMINATION USING THE COMBUSTION METHOD

This is an instrument method derived from the Dumas method as an alternative method to the mercury catalyst Kjeldahl method. It has two advantages: (1) less time is needed for nitrogen determination, and (2) hazardous and toxic chemicals are not utilized. Nitrogen freed by combustion at high temperature in pure oxygen is measured by thermal conductivity detection and converted to equivalent protein by an appropriate numerical factor (AOAC, 1999). This method is applicable to all flours, cereal grains, oilseed, and animal feeds. The following instruments are widely used for this purpose: Models FP-2000 and FP-520 (Leco Corp.) and Flash EA 1112 and NA-2500 (CE Elantech, Inc.). Both Leco models utilize the principle of Dumas method with slight modification in combustion mechanism.

Materials

Any instrument or device designed to measure nitrogen by combustion, equipped with the following:

Furnace: to maintain minimum operating temperature of 950°C for pyrolysis of sample in pure (99.9%) oxygen; some systems may require higher temperatures

Isolation system: to isolate liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector; device for converting all nitrogen oxide compound products (NO_x) to N_2 or measuring N as NO_2 may be required and included in the design

Detection system: to interpret detector response as % nitrogen (w/w); may include features such as calibration on standard material, blank determination,

and barometric pressure compensation (any required calibration must be based on theoretical % nitrogen in pure primary standard organic material such as EDTA)
Grinder: capable of grinding samples to pass No. 20 sieve
Analytical balance: accurate to 0.01 mg
Barometer: Hg type, readable to 0.1 mm

1. Use any instrument or device designed to measure nitrogen by combustion. Follow manufacturer's recommendations and instructions unique to each instrument for the safe operation of the instrument.

2. Calculate total nitrogen:

   Crude protein, % = %N × 6.25, or %N × 5.70 in case of wheat grains.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Catalyst mixture

Mix 16.7 g potassium sulfate, 0.6 g titanium dioxide, 0.01 g anhydrous copper sulfate, and 0.3 g pumice and grind fine in a crucible. The mixed catalyst powder is stable under usual laboratory storage conditions at room temperature. Avoid moisture to prevent from caking. Commercially premixed tablets are also available.

Methyl red indicator

Dissolve 1 g methyl red (sodium salt) in 100 ml methanol.
NOTE: *Store in dark at room temperature.*

Sodium hydroxide solution, concentrated

Dissolve ~450 g NaOH pellets or flakes (low N) in water, cool, and adjust the volume to 1 liter with water. Dilute to 1:l; or use solution with specific gravity ≥1.36.

Standard solutions

Prepare and accurately standardize 0.5 N hydrochloric or sulfuric acid and 0.1 N sodium hydroxide (*UNIT G2.1*). After standardizing both acid and base, also check one against the other.

COMMENTARY

Background Information

Kjeldahl method

This method was developed to replace the hazardous mercury catalyst required in the original mercuric oxide Kjeldahl method. It has been evaluated through an interlaboratory comparison of catalysts and has been adopted as the official replacement for the mercuric-oxide catalyzed Kjeldahl method. An inter-laboratory evaluation (Berner, 1990) indicated that this method (which uses the copper/titanium catalyst mixture) produces results more closely in agreement with the mercuric oxide catalyst method than methods using a copper sulfate catalyst. As a result of this study, mercuric oxide, copper sulfate, and Kjeldahl-Foss Automatic methods were declared obsolete in 1991.

The AOAC collaborative study (Kane, 1987) showed no bias when comparing Cu/Ti catalyst with HgO. The AOCS inter-laboratory evaluation (Berner, 1990), based on sample weight, acid, volume, digestion temperature, and time, previously specified in the mercury catalyst method, indicated an average bias of –0.244%, which was statistically significant at the 99% confidence level. Factors that may affect method bias are sample weight and particle size, catalyst composition, use of alundum, volume of acid used for digestion, digestion temperature, and digestion time. In 1991, two additional collaborative studies (cotton-

seed and cottonseed meal) showed that when 30 ml of sulfuric acid were used, caking of the digest was reduced and results were comparable to the mercuric oxide catalyzed method. As a result, the AOCS versions of this method may specify the use of >20 ml of sulfuric acid, but otherwise conform with the AOAC version (AOAC, 1999a).

Dumas method

The principle behind this method is based on mineralization (or calcination) of the sample in the presence of copper oxide. Carbon and hydrogen are oxidized in the form of CO_2 and water and then trapped. The volume of nitrogen released is measured, from which its content is deduced. In the beginning, this method was used to analyze microquantities of samples, but now instruments that can handle 1 g quantities of dry or wet samples are readily available. For this reason, this method is rapidly becoming one of preferred methods of nitrogen determination.

Combustion method

The performance of the combustion method compares favorably with that of the mercury catalyst Kjeldahl method for determination of crude protein in feeds. Standard deviations by the combustion method covered the full range of possibilities compared to the Kjeldahl method; values were either equivalent, better than, or not as good as for the Kjeldahl method. For the 0.5-mm pairs, values ranged from 0.09% to 0.58% protein for the Kjeldahl method and from 0.14% to 0.33% protein for the combustion method, and (S_R) values ranged from 0.23% to 0.86% protein (Kjeldahl) and from 0.30% to 0.61% protein (combustion).

The recovery data on standards demonstrates the ease with which the combustion method recovers nitrogen and emphasizes the difficulties of achieving full recovery with Kjeldahl methods. This has to be considered when comparing the two methods or making recommendations. The average difference between methods in the collaborative study was 0.04% N.

Critical Parameters

Kjeldahl method

Complete conversion of organic nitrogen to ammoniacal nitrogen is essential to obtain accurate and precise results. After the liquid has become clear and colorless, it may be necessary to digest the sample for an additional ~30 to 50 min. Reagent proportions, heat input, and digestion time are critical factors and should not be changed. Prolonged digestion and high temperatures must be avoided, because some ammoniacal nitrogen can be lost by oxidation. Large sample particles should be avoided, because the larger particles will require a longer digestion time.

The cooled digest should be liquid, or liquid with a few small crystals. Caking before the addition of water indicates too little residual acid at the end of the digestion period and may result in low nitrogen values.

More than 20 ml of concentrated sulfuric acid is needed for samples high in fat or oil, because more acid is required to effect complete digestion, avoid loss in nitrogen, and prevent caking. In recent collaborative studies involving the application of this method to cottonseed meal, it was observed that when 30 ml of H_2SO_4 were used, caking was reduced and results were comparable to the mercuric oxide catalyzed method.

Dumas method

The system must meet or exceed minimum performance specifications as follows.

a. Capable of measuring nitrogen in materials containing 0.2% to 20% nitrogen.

b. Demonstrate system accuracy based on 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations of nitrogen in lysine-HCl or tryptophan. Means of determinations must be ±0.15 of respective theoretical values, with standard deviations ≤0.15. System accuracy must not be tested with same material used for calibration.

Combustion method

The system must meet or exceed following minimum performance specifications.

a. System must be capable of measuring nitrogen in feed materials containing 0.2% to 20% nitrogen.

b. Accuracy of system is demonstrated by making 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations in lysine-HCl. Means of determinations must be within ±0.15 of the respective theoretical values, with standard deviations <0.15. Standard tryptophan may be substituted for lysine-HCl. System accuracy must not be tested with same material used for calibration.

c. Suitable fineness of grind is that which gives relative standard deviation (RSD) <2.0% for 10 successive determinations of nitrogen in mixture of corn grain and soybeans (2 + 1) that

has been ground for analysis. %RSD = (SD/mean %N) × 100. Fineness (i.e., ~0.5 mm) required to achieve this precision must be used for all mixed feeds and other nonhomogeneous materials.

d. Properly blanked system is demonstrated by analyzing suitable N Blank material, such as powdered cellulose, and obtaining zero reading. Atmospheric blank corrections with software (constants) introduce error and are not necessary when the system is properly configured (purged) and sample is properly introduced to minimize trapped atmospheric nitrogen.

Anticipated Results

The Kjeldahl method determines total nitrogen present in the sample. This includes protein nitrogen as well as nonprotein nitrogen. For this reason, this method tends to give a higher protein value than that actually present in the sample. This is not a problem for samples with relatively small amounts of non-protein nitrogen but care must be taken when analyzing samples with large amounts of nonprotein nitrogen sources.

With Dumas and Combustion methods, there are good agreements with the Kjeldahl determination; however, there are dangers of interference if the product is high in carbohydrates or sodium chloride.

Time Considerations

Kjeldahl method

On average, the Kjeldahl nitrogen determination method takes about 2 to 3 hr to complete a run—~70 to 90 min for sample digestion, 30 to 40 min for cooling, and 5 to 10 min for distillation and titration. In addition, the time needed to properly heat the heating block, ranging from 2 to 3 hr depending on the type, should be considered in advance of the analysis.

Dumas/Combustion methods

The major benefits of this method are the very rapid turnaround time, usually 5 min at a rate of ≤100 samples/day, and the elimination of contaminants associated with the use of mercury and copper catalysts.

Literature Cited

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AOAC, 1999c. Official Method 990.03. Ch. 4, p. 18 AOAC International, Gaithersburg, Md.

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Spectrophotometric Determination of Protein Concentration

This unit describes spectrophotometric methods for measuring the concentration of a sample protein in solution. These methods are most appropriate for purified proteins. Some food components may interfere (or absorb) at these wavelengths. In Basic Protocol 1, absorbance measured at 280 nm (A_{280}) is used to calculate protein concentration by comparison with a standard curve or published absorptivity values for that protein (a_{280}). In the Alternate Protocol, absorbance measured at 205 nm (A_{205}) is used to calculate the protein concentration. The A_{280} and A_{205} methods can be used to quantitate total protein in crude lysates and purified or partially purified protein. Both of these methods are simple and can be completed quickly. The A_{280} method is the most commonly used. The A_{205} method can detect lower concentrations of protein and is useful for dilute protein samples, but is more susceptible to interference by reagents in the protein sample than the A_{280} method. Basic Protocol 2 uses a spectrofluorometer or a filter fluorometer to measure the intrinsic fluorescence emission of a sample solution; this value is compared with the emissions from standard solutions to determine the sample concentration. The fluorescence emission method is used to quantitate purified protein. This simple method is useful for dilute protein samples and can be completed in a short amount of time.

USING A_{280} TO DETERMINE PROTEIN CONCENTRATION

BASIC PROTOCOL 1

Determination of protein concentration by measuring absorbance at 280 nm (A_{280}) is based on the absorbance of UV light by the aromatic amino acids tryptophan and tyrosine, and by cystine, disulfide bonded cysteine residues, in protein solutions. The measured absorbance of a protein sample solution is used to calculate the concentration either from its published absorptivity at 280 nm (a_{280}) or by comparison with a calibration curve prepared from measurements with standard protein solutions. This assay can be used to quantitate solutions with protein concentrations of 20 to 3000 µg/ml.

Materials

3 mg/ml standard protein solution (see recipe; optional)
Sample protein
Spectrophotometer with UV lamp

1. For calibrating with standards, use the 3 mg/ml standard protein solution to prepare dilutions of 20, 50, 100, 250, 500, 1000, 2000, and 3000 µg/ml in the same solvent as used to prepare the sample protein. Prepare a blank consisting of solvent alone.

   Ideally, for purified or partially purified protein, the protein standard should have an aromatic amino acid content similar to that of the sample protein. For the total protein of a crude lysate, bovine serum albumin (BSA) is a commonly used standard for spectrophotometric quantitation of protein concentration. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 280 nm. Allow the instrument to warm up 30 min before taking measurements.

3. Zero the spectrophotometer with the solvent blank.

4. Measure the absorbance of the protein standard and unknown solutions.

   If the A_{280} of the sample protein is >2.0, dilute the sample further in the same solvent and measure the A_{280} again.

Contributed by Michael H. Simonian
Current Protocols in Food Analytical Chemistry (2002) B1.3.1-B1.3.7

5a. *If the a_{280} of the protein is known:* Calculate the unknown sample concentration from its absorbance value using Equation B1.3.1.

In this equation a_{280} has units of ml/mg cm and b is the path length in cm.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280}}{a_{280} \times b}$$

Equation B1.3.1

5b. *If standard solutions are used for quantitation:* Create a calibration curve by either plotting or performing regression analysis of the A_{280} versus concentration of the standards. Use the absorbance of the sample protein to determine the concentration from the calibration curve.

ALTERNATE PROTOCOL

USING A_{205} TO DETERMINE PROTEIN CONCENTRATION

Determination of protein concentration by measurement of absorbance at 205 nm (A_{205}) is based on absorbance by the peptide bond. The concentration of a protein sample is determined from the measured absorbance and the absorptivity at 205 nm (a_{205}). This assay can be used to quantitate protein solutions with concentrations of 1 to 100 µg/ml protein.

Additional Materials *(also see Basic Protocol 1)*

Brij 35 solution: 0.01% (v/v) Brij 35 (Sigma) in an aqueous solution appropriate for dissolving or diluting the sample protein

1. Dissolve or dilute the protein sample in Brij 35 solution.

   Brij35 is used to prevent protein from adsorbing to glass and plastic surfaces, which can lead to significant errors when quantifying dilute solutions.

2. Turn on the UV lamp of the spectrophotometer and set the wavelength to 205 nm. Allow the instrument to warm up 30 min before taking measurements.
3. Zero the spectrophotometer with the Brij 35 solution alone.
4. Measure the absorbance of the sample protein.

5a. *If the a_{205} of the protein is known:* Use Equation B1.3.1 to calculate the concentration of the sample protein *except* substitute the appropriate values for A_{205} and a_{205}.

5b. *If the a_{205} is not known:* Estimate the concentration of the sample protein from its measured absorbance using Equation B1.3.2.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{205}}{31 \times b}$$

Equation B1.3.2

In this equation, the absorptivity value, 31, has units of ml/mg cm and b is the path length in cm.

USING FLUORESCENCE EMISSION TO DETERMINE PROTEIN CONCENTRATION

Protein concentration can also be determined by measuring the intrinsic fluorescence based on fluorescence emission by the aromatic amino acids tryptophan, tyrosine, and/or phenylalanine. Usually tryptophan fluorescence is measured. The fluorescence intensity of the protein sample solution is measured and the concentration is calculated from a calibration curve based on the fluorescence emission of standard solutions prepared from the purified protein. This assay can be used to quantitate protein solutions with concentrations of 5 to 50 µg/ml.

Materials

Protein standard solution prepared using the purified protein (see recipe)
Sample protein
Spectrofluorometer *or* filter fluorometer with an excitation cutoff filter ≤285 nm and an emission filter >320 nm

1. Prepare dilutions of the purified protein at 5, 7.5, 10, 25, and 50 µg/ml in the same solvent as the sample protein. Prepare a blank consisting of solvent alone.
2. Turn on the lamp of the instrument and allow it to warm up 30 min before taking measurements.

   If a spectrofluorometer is used, set the excitation wavelength to 280 nm and the emission wavelength to between 320 and 350 nm. If the exact emission wavelength is not known, determine it empirically by scanning the standard solution with the excitation wavelength set to 280 nm. If the instrument is a filter fluorometer, use an excitation cutoff filter ≤285 nm and an emission filter >320 nm.
3. Zero the instrument with the solvent blank.
4. Measure the fluorescence of the protein standard and sample protein solutions.
5. Create a calibration curve by either plotting or performing regression analysis of the fluorescence intensity versus concentration of the standards. Using the fluorescence intensity of the sample protein, determine the concentration from the calibration curve.

   Fluorescence emission is a linear function of concentration only over a limited range.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Standard protein solution, 3 mg/ml

Weigh out dry protein and prepare a stock solution at a concentration of 3 mg/ml in the same solvent as used for the sample protein. Store up to 3 months at −20°C.

To prepare calibration standard solutions, dilute the stock solution in solvent to give the desired final concentrations for the standard curve.

Bovine serum albumin (BSA, fraction V; Sigma) is frequently used for a protein standard solution. A 3 mg/ml solution of BSA should have an A_{280} of 1.98, based on an A_{280} of 6.61 for a 1% (w/v) solution.

For quantitation of a purified or partially purified protein, if possible, the protein standard should have an aromatic amino acid content similar to that of the sample protein.

COMMENTARY

Background Information

Measuring absorbance at 280 nm (A_{280}) is one of the oldest methods for determining protein concentration (Warburg and Christian, 1942; Layne, 1957). This method is still widely used because it is simple and does not require incubating the sample with exogenous chromophores. However, the detection limit is higher than colorimetric methods and therefore higher concentrations of protein are necessary. The A_{280} method requires that the protein being quantitated have aromatic amino acids, primarily tryptophan. Because of the variability in aromatic amino acid content among different proteins, their absorptivity at 280 nm (a_{280}) also varies. Therefore, if calibration standards are used for quantitation, the aromatic amino acid content of the standard must be similar to that of the sample protein for accurate results. For many previously studied proteins, the a_{280} is known and can be obtained from the literature (Fasman, 1989).

If the absorptivity is not known but the amino acid composition is known, the molar absorptivity at 280 nm (ε_{280}) can be predicted from Equation B1.3.3 (Pace et al., 1995):

$$\varepsilon_{280} = (\text{no. of Trp})(5500) + (\text{no. of Tyr})(1490) + (\text{no. of cystine})(125)$$

Equation B1.3.3

In this equation, the number of tryptophan (Trp), tyrosine (Tyr), and cystine residues are each multiplied by an estimated ε_{280} value for each residue. This equation was derived for folded proteins in water. With the DNA sequence for many proteins now available, the predicted ε_{280} can be derived for newer proteins where this value is not published. However, there are caveats that should be considered if the predicted ε_{280} in Equation B1.3.3 is used. First, the predicted ε_{280} is most reliable if the protein contains tryptophan residues and less so if there are no tryptophan residues. Second, the number of cystine residues must be accurately known and one cannot presume that all cysteine residues, to an even number, are involved in disulfide bonds. Alternatively, there are methods for measuring the ε_{280} value that are reliable (Pace et al., 1995).

Accordingly, the quantitation of proteins by peptide bond absorption at 205 nm (A_{205}) is more universally applicable among proteins. Furthermore, the absorptivity for a given protein at 205 nm is several-fold greater than that at 280 nm (Scopes, 1974; Stoscheck, 1990). Thus lower concentrations of protein can be quantitated with the A_{205} method. The disadvantage of this method is that some buffers and other components absorb at 205 nm (Stoscheck, 1990).

In addition to the aromatic amino acids, several others have absorption maxima in the UV range. Table B1.3.1 shows the wavelengths of absorption maxima and corresponding molar absorptivity (ε) for the amino acids with appreciable absorbance in the UV range. Only tryptophan has an absorption maximum at 280 nm,

Table B1.3.1 Absorption Maxima and Molar Absorptivity (ε) of Amino Acids[a]

Amino acid	Wavelength maxima (nm)	$\varepsilon \times 10^{-3}$ (l/mol cm)
Cysteine	250	0.3
Histidine	211	5.9
Phenylalanine	188	60.0
	206	9.3
	257	0.2
Tryptophan	219	47.0
	279	5.6
Tyrosine	193	48.0
	222	8.0
	275	1.4

[a]Values are for aqueous solutions at pH 7.1 (Freifelder, 1982; Fasman, 1989).

Table B1.3.2 Molar Absorptivity (ε) of Amino Acids at 280 nm[a]

Amino acid	$\varepsilon \times 10^{-3}$ (l/mol cm)
Cystine	0.110
Phenylalanine	0.0007
Tryptophan	5.559
Tyrosine	1.197

[a]Values are for aqueous solutions at pH 7.1 except for cystine, which is for water (Fasman, 1989).

Table B1.3.3 Fluorescence Properties of Aromatic Amino Acids[a]

Amino acid	Excitation wavelength	Emission wavelength	Quantum yield
Phenylalanine	260 nm	283 nm	0.04
Tryptophan	285 nm	360 nm	0.20
Tyrosine	275 nm	310 nm	0.21

[a]Values are for aqueous solutions at pH 7 and 25°C (Hawkins and Honigs, 1987; Fasman, 1989).

although, tyrosine and cystine will also slightly absorb. The ε_{280} for tryptophan is nearly five-fold greater than that for tyrosine and 50-fold greater than that for cystine (Table B1.3.2). The contribution to the absorbance at 280 nm of the third aromatic amino acid, phenylalanine, and cysteine are negligible. Several amino acids other than those in Table B1.3.1 absorb light below 205 nm (Fasman, 1989), but either the molar absorptivities are too low to be significant or the wavelengths are too short for practical absorbance measurements.

The aromatic amino acids also have fluorescence emissions when excited by light in the UV range. Table B1.3.3 gives the excitation wavelength, fluorescence emission wavelength, and quantum yield (Q) for tryptophan, tyrosine, and phenylalanine. The quantum yield is the ratio of photons emitted to photons absorbed. Typically, phenylalanine fluorescence is not detected in the presence of tyrosine and tryptophan due to low Q. Furthermore, tyrosine fluorescence is nearly completely quenched if the tyrosine residue is ionized or near an amino group, a carboxyl group, or a tryptophan residue (Teale, 1960; Freifelder, 1982). Therefore, tryptophan fluorescence is what is customarily measured.

Measurement of intrinsic fluorescence by aromatic amino acids is primarily used to obtain qualitative information (Freifelder, 1982). However, with a protein standard whose aromatic amino acid content is similar to that of the sample, intrinsic fluorescence can be used for quantitation (Hawkins and Honigs, 1987). An additional consideration is that the tertiary structure of a protein will influence the fluorescence, e.g., adjacent protonated acidic groups in a protein molecule will quench tryptophan fluorescence (Freifelder, 1982).

Critical Parameters and Troubleshooting

A 1-cm path length quartz cuvette is most often used to make absorbance measurements. However, quartz cuvettes with shorter path lengths, 0.01 to 0.5 cm, are available (e.g., from Hellma Cells or Beckman); these shorter path length cuvettes allow higher concentrations of protein solutions to be measured. Equation B1.3.1 and Equation B1.3.2 assume the cuvette has a path length of 1 cm; when cuvettes of shorter path length are used, the correct value for b must be substituted in the equation.

The solvent pH and polarity will affect the absorbance and fluorescence properties of a protein. A notable example of pH effects on absorbance is seen with tyrosine residues, where a change in pH from neutral to alkaline results in a shift of the absorbance maximum to a longer wavelength and an increase in absorptivity due to dissociation of the tyrosine phenolic hydroxyl group (Freifelder, 1982; Fasman, 1989). An example of solvent polarity effects on fluorescence is observed with tryptophan, where a decrease in solvent polarity

Table B1.3.4 Concentration Limits of Interfering Reagents for A_{205} and A_{280} Protein Assays[a]

Reagents[b]	A_{205}	A_{280}
Ammonium sulfate	9% (w/v)	>50% (w/v)
Brij 35	1% (v/v)	1% (v/v)
DTT	0.1 mM	3 mM
EDTA	0.2 mM	30 mM
Glycerol	5% (v/v)	40% (v/v)
KCl	50 mM	100 mM
2-ME	<10 mM	10 mM
NaCl	0.6 M	>1 M
NaOH	25 mM	>1 M
Phosphate buffer	50 mM	1 M
SDS	0.10% (w/v)	0.10% (w/v)
Sucrose	0.5 M	2 M
Tris buffer	40 mM	0.5 M
Triton X-100	<0.01% (v/v)	0.02% (v/v)
TCA	<1% (w/v)	10% (w/v)
Urea	<0.1 M	>1 M

[a]Values from Stoscheck (1990).

[b]Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

results in a shift in fluorescence emission to shorter wavelengths and an increase in intensity (Freifelder, 1982). Because of these effects, the following precautions should be taken for accurate results: (1) when calibration curves are used for quantitation by absorbance or fluorescence, standards must be in the same solvent as the samples; and (2) when a published absorptivity at a given wavelength is used for quantitation, the solvent composition of the sample must be the same as that used in obtaining the published data.

Various food components may interfere with absorbance readings at these wavelengths. For instance, plant phenolics absorb at 280 nm and can present a limitation to this method. This method should thus be used with purified protein solutions. Many buffers and other reagents used during purification can interfere with A_{280} and A_{205} spectrophotometric measurements. Stoscheck (1990) lists the concentration limits for many such reagents used in these spectrophotometric methods. The more commonly used reagents that absorb at 280 and 205 nm are listed in Table B1.3.4. In addition, reagents that contain carbon-carbon or carbon-oxygen double bonds can interfere with the A_{205} method.

Because stray light can affect the linearity of absorbance versus concentration, absorbance values >2.0 should not be used for sample proteins measured by the A_{280} or A_{205} method. Samples with absorbance >2.0 should be diluted further in the appropriate buffer to obtain absorbances <2.0.

Nucleic acids have substantial absorbance at 280 nm and can interfere with A_{280} quantitation of protein in crude samples. To resolve the protein concentration in such samples, measure the absorbance at 260 nm and 280 nm and calculate the protein concentration as follows (Warburg and Christian, 1942; Layne, 1957): protein concentration (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$. This estimation of protein concentration is valid up to 20% (w/v) nucleic acid or an A_{280}/A_{260} ratio <0.6.

Turbidity may lead to errors in absorbance as particulates may scatter light. If turbid samples must be used, the amount of turbidity needs to be consistent in all dilutions of the unknown sample and in any standards used for calibration. If this is not possible, the sample should be clarified by filtration or centrifugation.

Anticipated Results

Depending on the protein, the concentration range for the A_{280} method is 20 to 3000 µg/ml, for the A_{205} method is 1 to 100 µg/ml, and for the fluorescence emission method is 5 to 50 µg/ml.

Published absorptivities of proteins at 280 nm are usually given as the absorbance for a 1% (w/v) protein solution per cm, $A^{1\%}$, or as the molar absorptivity, ε, which has units of l/mol cm. To convert these published coefficients to units of mg/ml, use either Equation B1.3.4 or Equation B1.3.5.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280} \times 10}{A^{1\%} \times b}$$

Equation B1.3.4

Molecular weight in Equation B1.3.5 is the molecular weight of the protein.

$$\text{concentration}\left(\text{mg/ml}\right) = \frac{A_{280} \times \text{molecular weight}}{\varepsilon_{280} \times b}$$

Equation B1.3.5

Time Considerations

When the absorptivity for a protein is known, the A_{280} and A_{205} measurements require <30 min depending on the number of samples. When standards are used for quantitation with these assays or for intrinsic fluorescence quantitation, 1 hr is required.

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Detailed discussion of intrinsic fluorescence of proteins and factors that affect fluorescence emission by the aromatic amino acids (see pp. 618-663).

Fasman, 1989. See above.

Contains tables with absorptivities for UV spectrophotometric detection and tables with data on excitation and emission wavelengths for fluorescence detection of many proteins. Also includes a table with molecular weights for many characterized proteins.

Stoscheck, 1990. See above.

Contains a list of substances that can interfere with 205- and 280-nm spectrophotometric measurements of proteins and of concentration limits for these substances.

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Analyses of Protein Quality

Determining protein quality analysis is important in food science, particularly for developing foods with targeted nutritional value, and in animal feeding and husbandry. Protein is the key component in the diet of any farmed species, particularly in aquaculture and the pet food industry, making an accurate assessment of protein utilization critically important. Protein quality analysis provides an estimate of the content and bioavailability of indispensable or dietary essential amino acids.

A number of procedures used to determine protein quality involve bioassays. Bioassays require feeding live animals protein ingredients for a specified period of time, and then estimating the nutritive value of the protein. Two such assays are the rat-based protein efficiency ratio (PER) bioassay and the human nitrogen balance assay (Dimes et al., 1994). Animal feeding experiments require chemical analyses of both the dietary "inputs" and then the metabolic "output" of the animal (e.g., body composition analysis, fecal sample analysis, collection, and assay for urine) from which the efficiency of protein metabolism can be predicted as well as how the protein supports animal growth and cell maintenance.

USING PROTEIN EFFICIENCY RATIO (PER) TO DETERMINE PROTEIN QUALITY

BASIC PROTOCOL 1

PER is a method to metabolize or determine the quality of protein in foods. Quality is measured by the amount of usable protein and the growth resulting from it through an animal assay. Formerly, this method was used as the standard method for all protein quality analysis. However, there is some question as to whether or not it is a valid measurement. This is because PER does not account for the differences in amino acid requirements between humans and rats (Seligson and Mackey, 1984), nor does PER account for the protein needed for cell maintenance. Therefore, PER results often overestimate the requirements for some amino acids and underestimate others. Specifically, PER tends to underestimate the protein quality of lysine-deficient proteins such as wheat gluten (Hackler, 1977).

However, since the PER is an in vivo test, protein digestibility and amino acid bioavailability are encompassed to some extent within the assay. Despite these advantages, it is difficult to determine the individual contribution of digestibility and bioavailability of individual amino acids, or of individual proteins in a complex mixture, on overall protein quality. There are indications that the assay can be shortened from 4 weeks to 2 weeks with little loss in accuracy (Hackler, 1977).

Materials

A minimum of 10 rats per assay group (i.e., 10 control group, 10 test group): male weanlings (Sprague-Dawley), 50 to 70 g from the same colony, 21 to 28 days old

PER control group diet (see formulas provided in Critical Parameters; casein is assigned a PER of 2.5; results for the test protein or proteins are normalized against this value in an attempt to reduce interlaboratory variation)

PER test group diet (see formulas provided in Critical Parameters)

Commercial laboratory rat chow

Diet to be tested

Individual stainless steel screen-bottom cages with removable food cups

Animal room maintained at 18° to 26°C, 40% to 70% relative humidity, and 12 hr light/dark cycle

Contributed by Barbara Rasco

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1. House animals in individual stainless steel screen-bottom cages in a room maintained at 18° to 26°C with a 12 hr light/dark cycle and 40% to 70% relative humidity. Provide food and water ad libitum.
2. For a 2-day acclimation period, feed the rats a commercial laboratory rat chow diet.
3. Record weight of each animal before test trial begins.

   Initial weight of rats should be between 50 and 70 g. Distribute rats between diet treatment groups so that the average weight and range of weights will be similar for each of the test groups.

4. Feed rats the specified diet for a 28-day trial period.

   Rats are fed the test diet ad libitum. Rats on the reference protein (control) diet are also fed ad libitum.

5. Two times a week, remove and weigh the food cups. Fill with fresh diet and reweigh prior to placement back into the cage. Change water in water bottle.
6. Record body weight of each rat every 7 days, and at the end of the trial period.
7. Calculate the PER based on weight gained by test group in grams divided by the total amount of protein consumed (*UNIT B1.2*). Total protein is calculated based upon measurement of total nitrogen in the diet.

   The final PER value is an average *of the weight gain and protein intake of the entire test group at day 28.*

$$\text{PER} = \frac{\text{weight gain of test group (g)}}{\text{total protein consumed (g)}}$$

8. Calculate the relative PER (RPER).

   RPER provides a value for the test protein relative to casein when casein is run as part of the same experiment.

$$\text{RPER} = \frac{\text{PER of the test protein} \times 2.5}{\text{PER of casein}}$$

ALTERNATE PROTOCOL 1

PER INCORPORATING A NET PROTEIN RATIO CORRECTION

Net protein ratio (NPR) is used to correct PER values for the amount of protein required for cell maintenance. NPR is often run in conjunction with a PER. The experiment requires that one additional set of animals be added as a treatment group. This group of animals is fed a basal diet with no protein (zero protein or basal diet). Results from RNPR are similar to net protein utilization (NPU) and biological value (BV methods, see Alternate Protocol 4). A 2-week RNPR is thought to be the most appropriate rat test for routine assessment of protein quality.

Additional Materials *(also see Basic Protocol 1)*

Additional group of experimental animals
Basal diet with no protein

1. Follow the steps in Basic Protocol 1, but include an additional group of animals fed a basal diet with no protein.

2. Take the initial weight of each animal in the zero protein group at day 1 and the weight of the animals at regular intervals.
3. Record the average weight loss of the animals in the zero protein group for day 10 and 14.
4. Calculate RNPR using the following equations:

$$\text{NPR} = \frac{\text{avg. wt. gain of test group (g)} + \text{avg. wt. loss of basal diet group}}{\text{protein consumed by test group (g)}}$$

$$\text{RNPR} = \frac{\text{NPR test protein}}{\text{NPR reference protein (casein)}} \times 100$$

IN VIVO OR TRUE PROTEIN DIGESTIBILITY TO DETERMINE PROTEIN QUALITY

ALTERNATE PROTOCOL 2

In vivo protein digestibility assays can be conducted separately as part of a protein digestibility–corrected amino acid score (PDCAAS, see Basic Protocol 2) experiment for estimating true protein digestibility or in conjunction with PER assays (using the same groups of rats as the PER experiment, see Alternate Protocol 3). The method given in this protocol is the recommended method. In vivo (true) protein digestibility assays are based upon measurement of the amount of protein not absorbed but passed through into the feces. The weight and nitrogen content of the feces are determined by a Kjeldahl method (*UNIT B1.2*). The feed intake for the same period and nitrogen content is determined as outlined below.

Additional Materials *(also see Basic Protocol 1 and Alternate Protocol 1)*

Moderately absorbent paper (e.g., filter paper)

1. Line the cages with a moderately absorbent paper such as filter paper.
2. Distribute male weanling rats between treatment groups (i.e., 4 rats per group, 2 groups per treatment).

   Weights between the rats within each group should not vary by >5 g.

3. Feed rats a commercial rat chow diet for a 2-day acclimation period.
4. Reweigh animals before the experiment begins. Feed animals the test diets at a level of 15 g dry matter/day. Feed animals in a protein-free test group the test diets for a total of 9 days.
5. Collect feces on each of the last five days of the test. Also collect spilled food, so an accurate measurement of feed consumption can be calculated for each rat.
6. Allow the spilled, uneaten food to air dry for 3 days before measuring the weight.
7. Determine the total food consumption by subtracting the amount of food spilled from the 15 g provided per day.
8. Dry the feces in a vacuum oven overnight, or lyophilize and grind, for nitrogen analysis using a Kjeldahl method (*UNIT B1.2*).

   For metabolic fecal nitrogen determination, fecal nitrogen from a zero-protein test group must also be determined.

9. Calculate the true protein digestibility using the following equation:

$$\text{True protein digestibility} = \{[PI - (FP - MFP)]/PI\} \times 100$$

where PI equals protein intake (g); FP is fecal protein (g); and MFP is the metabolic fecal protein (g).

The MFP is calculated from the amount of protein in the feces of rats fed a protein-free diet (Sarwar, 1996).

ALTERNATE PROTOCOL 3

PER AND IN VIVO PROTEIN DIGESTIBILITY

Alternate Protocol 3 is a variation of Alternate Protocol 2 and describes a method for measuring in vivo protein digestibility that is run in conjunction with the PER assay.

See Basic Protocol 1 for materials.

1. Begin this Alternate Protocol at Basic Protocol 1, step 1.
2. Collect the feces for each rat in the PER assay during days 18 to 28 of the PER experiment and store frozen in sealed containers.
3. Record the feed intake during the last 4 days of the PER experiment.
4. Pool the collected feces from all rats from each diet group, lyophilize them, grind, and weigh.
5. Measure the nitrogen content of the feces using a Kjeldahl method as described in *UNIT B1.2*. For metabolic fecal nitrogen determination, measure the fecal nitrogen content from a zero-protein test group (see Basic Protocol 1).
6. Calculate true protein digestibility using the equation in Alternate Protocol 2, step 9.

ALTERNATE PROTOCOL 4

NET PROTEIN UTILIZATION (NPU) AND BIOLOGICAL VALUE (BV) TO DETERMINE PROTEIN QUALITY

An NPU assay can be conducted in conjunction with a PER assay. NPU is used as a means of predicting the biological value of a protein. For NPU assays, the protein content of the animal carcasses is determined. Biological value (BV) measures the efficiency of the utilization of nitrogen by the animal. The biological value of a test protein is the proportion of nitrogen that is retained for maintenance and for growth, corrected for metabolic and endogeneous losses of nitrogen (BV= N retained/N absorbed). Retained N refers to the nitrogen retained by the body for growth and maintenance, while absorbed N refers to that which enters the lumen (generally in the form of protein), and is hydrolyzed and absorbed. However, these are extremely difficult assays to conduct reliably. The protein level in the diet and animal age will affect the results of either a BV or an NPU assay. Also, NPU assays fail to account for the differential rate of utilization or reutilization of dietary essential amino acids. A protein deficient in an essential amino acid should have an NPU of zero. However, animals have mechanisms for salvaging and reutilizing endogeneous amino acids. For example, in the rat, lysine-deficient proteins have an NPU ranging from 30 to 50. Because of the extreme care needed to completely collect the urine and feces for the assay, prevent feed contamination in the urine and feces, determine metabolic fecal losses, and measure endogeneous urinary losses (Hackler, 1977), BV is calculated from the results of an NPU assay.

Additional Materials *(also see Basic Protocol 1)*

0.5 M Na_2SO_4
Cheesecloth
Rubber mallet or other hard tool
Commercial blender

1. Perform PER assay as described in Basic Protocol 1, establishing a test group, a control group, and a zero-protein group.
2. Sacrifice a minimum of 2 animals from each test group at the termination of the PER study using a blow to the head. Wrap each animal in tared cheesecloth, and then freeze whole at –30° to –40°C for a minimum of 48 hr.
3. Fracture the carcass with a rubber mallet or other hard tool into pieces not >3 cm in each dimension and freeze dry (<50 mTorr) for 3 days or until carcass fragments are completely lyophilized.
4. Homogenize each lyophilized carcass in 3 volumes of 0.5 M Na_2SO_4 in a commercial blender. Take a minimum of 3 samples from each homogenate for analysis (Dong et al., 1990).
5. Calculate the net protein utilization (NPU), which is the proportion of nitrogen intake that is retained as is equivalent to BV × true digestibility, by comparing the carcass nitrogen content of a group of animals fed the test-protein diet to that of animals fed a zero-protein diet:

$$\text{NPU} = \frac{\text{N retained}}{\text{N intake}} = \text{BV} \times \text{true digestibility}$$

$$\text{NPU} = \frac{\text{body N of test group} - \text{body N of zero-protein group} + \text{N consumed by zero-protein group}}{\text{N consumed by test group}}$$

$$\text{BV} = \frac{\text{retained N}}{\text{absorbed N}} \times 100$$

$$\text{BV} = \frac{\text{N intake} - (\text{fecal N} - \text{metabolic N}) - (\text{urine N} - \text{endogeneous urinary N})}{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})} \times 100$$

NOTE: *See UNIT B1.2 for determination of N.*

PROTEIN DIGESTIBILITY–CORRECTED AMINO ACID SCORE (PDCAAS) TO DETERMINE PROTEIN QUALITY

BASIC PROTOCOL 2

The protein digestibility-corrected amino acid score (PDCAAS) has been adopted as a simpler approach for determining protein quality on a routine basis. No animal feeding studies are required. An additional advantage is that the method can often more closely predict the protein quality of a protein ingredient for humans than can assays in the growing rat. It is the method of choice for highly digestible proteins that contain minimal amounts of antinutritional factors.

PDCAAS (%) is calculated as the product of the true protein digestibility and the amino acid score (or lowest amino acid ratio) relative to reference protein. Amino acid composition of protein is determined by hydrolyzing the protein into its component amino acids and then separating the amino acids chromatographically as described below. True protein digestibility is determined according to Alternate Protocol 2.

Table B2.1.1 An Example Of Calculation For Amino Acid Score Using Whey Protein[a]

Amino acid	Whey (mg/g protein)	Reference protein values for preschool children (see Table B2.1.2)	Ratio (test protein/ref)
Isoleucine	17.8	28	0.64
Leucine	46	66	0.70
Lysine	44	58	0.76
Met + Cys	20.3	25	0.81
Phe + Tyr	27.4	63	0.43
Threonine	32.9	34	0.97
Tryptophan	9.2	11	0.84
Valine	28.1	35	0.80
Histidine	9	19	0.47

[a]Amino acid score = $(0.64 \times 0.7 \times 0.76 \times 0.81 \times 0.43 \times 0.97 \times 0.84 \times 0.8 \times 0.47)^{1/9} = 0.84$.

Materials

Sample containing ~10 mg nitrogen
6 M HCl
Amino acid derivatization reagents
Performic acid

1.5- to 2-ml nitrogen-purged ampule
Nitrogen-free filter

Determine amino acid composition of protein

1. Digest a sample containing ~10 mg N in 400 µl 6 M HCl refluxed at 100°C for 24 hr in a sealed 1.5- to 2-ml nitrogen-purged ampule. Collect the digest, filter through a nitrogen-free filter, and dilute to 500 µl with distilled, deionized water.

   Although acids, alkalis, or enzymes can be used for protein digestion, the most common digestion method involves heating the peptide or protein in concentrated hydrochloric acid.

2. Evaporate a 5-µl aliquot to dryness under a vacuum. Redissolve the residue in a suitable buffer prior to analysis by an amino acid analyzer or into the appropriate eluant if high performance liquid chromatography (HPLC) analysis is used.

3. Derivatize amino acids, either before (pre-column) or after (post-column) chromatography.

   During derivatization, amino acids are reacted with a dye to form UV-absorbent or fluorescent derivatives.

4. Oxidize methionine and cysteine to methionine sulfone and cysteic acid using performic acid prior to acid hydrolysis.

   Incomplete oxidation can be a problem. Higher recoveries of cysteine and cystine have been achieved by reduction of those amino acids with 2-mercaptoethanol followed by incubation with 4-vinylpyridine. This converts cysteine and cystine to S-(4-pyridylethyl)-L-cysteine, a derivative that can be separated by ion-exchange chromatography. Performic acid oxidation of methionine in the presence of phenol is a suitable method for analysis of cysteine.

   A single performic acid digestion can be used for all amino acids except tyrosine and tryptophan (Williams, 1982).

5. Calculate the amino acid score from an amino acid profile:

$$\text{amino acid score} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in 1 g of reference protein}}$$

See Table B2.1.1 for an example of the calculation for amino acid score using whey protein.

The limiting amino acid can be determined by taking the ratio of the amino acid present in the test protein and dividing it by the amount in the reference protein. The amino acid with the lowest ratio (test protein/reference) is the limiting amino acid. In this example, the single limiting amino acid is histidine (a dietary essential amino acid for young children). The lowest ratio is for the sum of phenylalanine and tyrosine.

6. Correct the amino acid score for protein digestibility by multiplying the lowest amino acid ratio with true protein digestibility (see Alternate Protocol 2). An example:

whey protein true digestibility = 0.95

corrected digestibility = (lowest amino acid ratio) × (true digestibility) = 0.43 × 0.95 = 0.41

IN VITRO PROTEIN DIGESTIBILITY ASSAY

BASIC PROTOCOL 3

The protein digestibility values for pH-shift methods generally correlate well with in vivo digestibility methods in the sense that both tests yield the same relative ranking for test proteins (Pedersen and Eggum, 1983). The difficulty with an in vitro method is that it does not accurately estimate qualitative differences between samples with low and high protein digestibilities.

Materials

Porcine pancreatic trypsin (Type IX)
Bovine pancreatic α-chymotrypsin (Type II)
Porcine intestinal peptidase (Grade I)
0.1 N NaOH or HCl
1 mg N/ml sodium caseinate (see recipe)
Test protein

37°C bath
0°C ice bath

NOTE: Where distilled water is called for in this protocol, glass-distilled is preferred.

1. Prepare enzyme solution fresh and keep on ice. Disperse the enzymes in distilled water at final concentrations: ~1.6 mg/ml trypsin (23,100 U), ~3.1 mg/ml chymotrypsin (186 U), and ~1.3 mg/ml peptidase (0.052 U).
2. Adjust the pH of the enzyme solution to 8.0 with 0.1 N NaOH or HCl. Warm exactly 2 min at 37°C and immediately place in a 0°C ice bath.
3. Prepare a solution of 1 mg N/ml sodium caseinate in distilled water.

   Use this solution as a standard to check the activity of the enzyme solution.

4. For the test protein, disperse 6.25 mg protein (1.0 mg nitrogen) in 10 ml distilled water.

   The nitrogen content of the test protein can be determined by Kjeldahl method (see UNIT B1.2). Adjust pH of suspension with 0.1 N NaOH while stirring suspension at 37°C.

5. Incubate protein suspension at 4°C for a minimum of 1 hr, but no more than 24 hr.
6. For casein standard, transfer 10 ml of sodium caseinate solution into a stirred reaction vessel. Warm vessel to 37°C.

7. Adjust pH to 8.0 with either 0.1 N NaOH or 0.1 N HCl depending on the pH of the solution and incubate for 5 to 10 min at 37°C.
8. Add 1.0 ml of the enzyme solution (prepared in step 1).
9. Stir solution constantly and hold at pH 7.98 for exactly 10 min. Record amount of 0.1 N NaOH needed to maintain a constant pH during this period.
10. Calculate true digestibility (TD) by using the following equation (Adler-Nissen, 1984 and 1986):

$$TD = 76.14 + 44.77B$$

where B = amount of 0.1 N NaOH used.

IMPORTANT NOTE: *Sodium caseinate values for protein digestibility should be 98% to 102%.*

11. Repeat steps 5 through 10, using exactly 10 mg N test protein dissolved in 10 ml distilled water.
12. Calculate the lab correction factor using the equation:

$$\text{lab correction factor} = \frac{100}{\text{sodium caseinate digestibility}}$$

The lab correction factor compensates for differences in proteolytic enzyme preparations and other experimental variables. Multiply the experimentally determined test protein digestibility by the correction factor to determine digestibility.

ALTERNATE PROTOCOL 5

IN VITRO PROTEIN DIGESTIBILITY FOR C-PER (AOAC METHOD 43.265)

C-PER is a PER calculated from the amino acid composition of the test protein and an in vitro protein digestibility measurement. Its calculation involves complicated algorithms provided as part of AOAC procedures.

Additional Materials *(also see Basic Protocol 3)*

Bacterial protease (Pronase P or E)
55°C water bath

1. Prepare these two enzyme solutions as per AOAC 43.261:

   Solution A: Make up fresh and store on ice. Dissolve 227,040 BAEE units of trypsin and 1860 BAEE units of α-chymotrypsin, 0.520 L-leucine β-napthylamide units of peptidase in 10 ml water.

   Solution B: Make up fresh and store on ice. Dissolve 65 casein units of bacterial protease in 10 ml water. Store on ice.

2. Add a solution of 1 mg N/ml sodium caseinate in distilled water to a small reaction vessel or vial and stir for 1 hr using a magnetic stirrer.
3. For the test protein, disperse 6.25 mg protein (1.0 mg nitrogen as determined by Kjeldahl method; see *UNIT B1.2*) in 10 ml distilled water into a small reaction vessel or vial. Adjust the pH of the suspension with 0.1 N NaOH while stirring at 37°C.
4. Calibrate pH meter at 37°C. Equilibrate casein solution (control) to pH 8 ± 0.03 by adding either dilute NaOH or HCl. Repeat for protein test solutions (see Basic Protocol 3). Hold both control and test protein solutions at 37°C.

5. Equilibrate enzyme solution A and enzyme solution B to pH 8 ± 0.03 by adding either dilute NaOH or HCl. Replace enzyme solutions on ice.
6. Add 1 ml enzyme solution A to reaction vessel with control sample and incubate exactly 10 min at 37°C.
7. Transfer reaction vessel to a 55°C water bath and incubate for exactly 19 min.
8. Transfer reaction back to the 37°C bath, insert pH electrode, and read pH at exactly 20 min.

   The pH at 20 min is X.
9. Calculate % digestibility as (Satterlee et al., 1979):

   % digestibility = 234.84 – 22.56 (*X*)
10. Repeat steps 5 to 9 for test proteins and calculate % digestibility as per equation in step 9.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Sodium caseinate, 1 mg N/ml

Suspend 10 ANRC reference casein (Humko Sheffield Chemical, Teklad Test Diets, or equivalent) in 200 ml water and adjust pH to 8.0 with NaOH. Maintain at pH 8 for at ≥1 hr. Freeze dry and determine nitrogen using Kjeldahl method (*UNIT B1.2*).

COMMENTARY

Background Information

Protein efficiency ratio (PER, see Basic Protocol 1) is an animal growth assay that uses rats as test subjects. Two groups of rats are fed a diet consisting either of the test protein or the reference protein, which is usually casein. The rats are fed for a specified period of time with weight gain and food consumption measured. PER was formerly the standard assay for all protein quality analysis; however uncertainties regarding the reliability of this rat growth assay to accurately predict the quality of a protein source in human nutrition resulted in its replacement with other tests. Because PER is a biological assay, it can provide an indication of protein requirements for cell maintenance, and protein digestibility if other tests are conducted along with it. For ranking protein quality, PER results correlate well with biological value (BV) and net protein utilization (NPU) tests (see Alternate Protocol 4). A modification of the PER that takes maintenance into account by comparing the growth between a lactalbumin control group and test protein groups is the relative nitrogen utilization (RNU) test. This method is an improvement over NPR, since NPR, which uses casein as a reference protein, tends to overestimate the protein quality for lysine-deficient proteins (Hackler, 1977).

An in vivo protein digestibility assay is often conducted in conjunction with a PER (see Alternate Protocol 3). For protein digestibility, the nitrogen ingested in the food is compared with that recovered in the feces (Dong et al., 1987). In vitro protein digestibility (see Basic Protocol 3 and Alternate Protocol 5) can be determined by incubating the test protein with a mixture of proteolytic enzymes and monitoring the degree of hydrolysis (Dimes et al., 1994).

The PER has been replaced by tests that determine a chemical score for a protein (see Basic Protocol 2). In this method, the biological value of a protein, including the limiting amino acid(s), can be predicted by comparing the amino acid profile of a test protein to that of a reference protein. The problem with chemical scoring methods is that they do not take into account how digestible a protein is for animals or people, nor do they predict whether amino acids are bioavailable or whether the test protein contains antinutritional or toxic factors, let alone whether the test protein is palatable as a sole or primary source of dietary protein. A related problem with the PDCAAS is that it

does not reflect the higher nutritional value of a protein that scores higher than the reference protein. Also, because it is an in vitro determination, PDCAAS cannot account for adverse antinutritional factors in a protein ingredient such as those present in plants, i.e., glucosinolates (mustard and canola), gossypol (cottonseed), phytates (cereals and oilseeds), hemagglutins and trypsin inhibitors (legumes). Neither can it account for antinutritional factors formed during heat processing or via chemical additions (e.g., acids, oxidizing agents). The method does not determine the true digestibility of poorly digestible or low-quality proteins with supplemental amino acids added; the bioavailability of individual amino acids may be as much as 44% lower than the overall digestibility of protein in the same food product (Sarwar, 1996). Because of this limitation, it is difficult to determine the actual limiting amino acid in a protein, and this affects the accuracy of the overall protein score.

Additional factors that may affect the reliability of the chemical scoring methods lie with the inherent difficulties of amino acid analysis. The analytical procedure for amino acid analysis can affect both the recovery and reliable quantitation of amino acids. Proteins must first be hydrolyzed to amino acids before analysis. Hydrolysis methods affect the amino acid recovery. Cystine, methionine, tryptophan, threonine, serine, and tyrosine can be destroyed during hydrolysis. Valine and isoleucine are released slowly and may not be completely recovered. There can also be significant differences in the amino acid profile for the same protein, depending upon the chromatographic methods chosen for amino acid analysis (Seligson and Mackey, 1984).

The most common chemical scoring method is the protein digestibility-corrected amino acid score (PDCAAS; see Basic Protocol 2). This method has been adopted by the FDA for nutritional labeling of foods in the United States and by FAO/WHO for routine analysis of protein quality for humans (Sarwar, 1996). A nutritionally complete protein is used as the reference protein for scoring protein quality. There are different scoring patterns for infants (which use the amino acid composition of human milk), preschool-aged children (2 to 5 years), school-aged children (10 to 12 years), and adults (see Table B2.1.2). The PDCAAS corrects the chemical score for protein digestibility. Digestibility is determined through an in vitro method. These in vitro methods use a series of enzymes that simulate the mix of digestive proteolytic enzymes in the small intestine.

In vitro protein digestibility assays provide an estimate of how a protein is digested, absorbed, and utilized. Differences in protein digestibility arise from the varying susceptibility of protein to enzymic hydrolysis in the digestive system. These differences can arise from processing treatments, such as thermal abuse, which reduce protein digestibility. These deleterious changes are due to alteration in the

Table B2.1.2 Suggested Amino Acid Profiles for Reference Proteins[a]

Amino acid (mg/g crude protein)	Age category			
	Infant	Pre-school 2-5 year	School age 10-12 year	Adult
Histidine	26(18-36)	19[b]	19[b]	16
Isoleucine	46(41-53)	28	28	13
Leucine	93(83-107)	66	44	19
Lusine	66(53-76)	58	44	16
Methionine + cystine	42(29-60)	25	22	17
Phenylalanine + tyrosine	72(68-118)	63	22	19
Threonine	43(40-45)	34	28	9
Tryptophan	17(16-17)	11	9[a]	5
Valine	55(44-77)	35	25	13
Total				
Including histidine	460(408-588)	339	241	127
Minus histidine	434(390-552)	320	222	111

[a]Adapted from: Comparison of suggested patterns of amino acid requirements with the composition of high quality animal proteins (Joint FAO/WHO Expert Consultation, 1991).
[b]Values interpolated from smoothed curves of requirements versus age.

primary and secondary structures of a protein through formation of chemical bonds that are not susceptible to digestive enzymes. How a protein has been treated is also important because processing or storage under abusive conditions can alter the three-dimensional structure of the protein, either improving or lessening its susceptibility to digestive enzymes. The presence of nonprotein dietary constituents can also affect how a protein is digested. Some of these components include phytate, dietary fiber, and various toxigenic agents. Amino acid digestibility can differ from the digestibility of the protein in a food. For certain foods such as legumes, the true digestibility of methionine, cystine, and tryptophan are 25% to 44% lower than the respective protein. However, differences between protein and amino acid digestibility for most proteins do not differ by >10%, making a correction of amino acid scoring using total protein digestibility sufficiently accurate.

In vitro digestibility assays are commonly conducted as part of a PDCAAS assay or in conjunction with the PER animal bioassay. Enzymes for the standard in vitro digestibility assay include porcine pancreatic trypsin, porcine intestinal peptidase, bovine pancreatic α-chymotrypsin, and often one or more bacterial proteases. The digestibility of the test protein is calculated relative to casein and is based upon the drop in pH resulting from protein hydrolysis. For this reason, these assays are called pH-shift procedures. One of the more popular in vitro digestibility assays is the three-enzyme procedure, which is the basis for the standard AOAC method 982.30. In vitro assays underestimate the digestibility of high-quality proteins such as dried egg and nonfat dry milk. The major limitation of the pH shift methods is that the pH is not constant during the course of the assay. Also, the buffering capacity of peptides, proteins, and other substances in the test protein may influence the pH to decline in a pH shift assay. Recently developed pH-stat methods are a more accurate means for measuring protein digestibility because the reaction pH can be controlled during the incubation period (Hsu et al., 1977; Satterlee et al., 1979; Dimes et al., 1994).

The advantages of an animal bioassay are that protein digestibility, amino acid bioavailability, and the presence of antinutritional factors can be ascertained. However, animal-based feeding studies can be difficult. First, these assays are expensive to conduct and time-consuming. Secondly, these assays require animals to be fed artificial diets that do not reflect what the animal would consume on a regular basis. For certain species, there are additional difficulties. In aquaculture, for example, collection of feces is difficult since these are dispersed in the water column and dissolve; urine collection is not possible without resorting to a system that restrains and stresses the fish, affecting its feeding behavior. Also, extrapolation of the results from animal feeding experiment, such as the PER bioassay to humans has been criticized because of the difference in amino acid requirements and growth rates between rats and people (Seligson and Mackey, 1984). Factors that affect the results of protein quality assays using animals include (Hackler, 1977): age and gender of animal, animal weight, protein quality and quantity, food intake, other dietary components in the feed (i.e., minerals, fat, carbohydrate, and moisture), animal husbandry practices, and environmental conditions (i.e., temperature, humidity, cage size, and lighting/photoperiod).

The interpretation of results from protein-quality assays for foods should be cautiously interpreted. For example, the palatability of ingredients is a function of compounds inherent in the ingredient or a result of formation of off-flavors resulting from processing (e.g., thermal abuse creating browning reaction products). Since protein-quality assessments are conducted with the test ingredient being the sole source of dietary protein, often fed to animals for a significant period of time, low palatability can confound a study and lead to unreliable predictions of protein quality. Low palatability has been observed in aquaculture diets containing defatted soybeans, thermally abused fish meal, and co-dried ensiled mixed protein ingredients.

Critical Parameters

Diet formula

1. *PER control group diet formula.* The protein component in the diet for the control group should be pure (reference) casein (ANRC reference casein, Teklab Diets). Casein is assigned a PER of 2.5. Results for the test protein(s) are normalized against this value in an attempt to reduce interlaboratory variation. The diet must be isocaloric (see recipe for PER Test Group Diet, below).

2. *PER test group diet formula.* Diet for test group with the protein fraction for the diet being the protein of interest (i.e., test protein): Test diets are 10% protein. The protein ingredient

must contain 1.6% nitrogen if it is to be incorporated into the test diet at the proper level. Diets must be <10% moisture. The fat content of high-fat foods should be reduced to <10% by ether extraction.

Diets are isocaloric. The composition of the test and control (reference protein) diet (calculated on a dry weight basis) is 10% protein (1.6% nitrogen), 1% AIN vitamin mix 76, 3.5% AIN mineral mixture 76 (Nutritional Biochemicals), 0.2% choline bitartrate, 5% cellulose (only if test food is <5% total dietary fiber), corn oil to 10% total fat, and corn starch to total 100%. To account for differences in the protein content of the test diet, the level of corn starch can be adjusted (Joint FAO/WHO Expert Consultation, 1991). The chemical composition (proximate analysis) of the test protein must be measured before test diets are formulated. The proximate analysis of the test and control diets are to be measured after the diets are formulated, but before they are fed, to ensure that the protein content is the same for all diets, and that the diets are isocaloric.

3. *PER zero-protein diet formula.* For determination of net protein ratio, a test diet that contains no protein is run as one of the test diets. This zero protein diet is used to derive a correction to account for the amount of protein required for cell maintenance.

PER

Accurate diet preparation and analysis are critical for any feeding study. The diet must be chemically uniform from the beginning to the end of the study, and the composition of each diet treatment must be accurately conducted. Experimentally determined differences between diet treatments will be reliable only if the composition of the diets can be relied upon—do the different treatments have the same protein and calorie content? Representative sampling of diets for testing is a prerequisite, as well as experience conducting an accurate proximate analysis. Palatability of the diets is also an issue in some studies, as well as the ease the animal has in consuming the diet. Proper acclimation of the animals prior to the study, and the overall health and well-being of the animals during the course of the study, are important to study outcome. Lethargy and failure to eat are not uncommon, and reasons for this must be determined, as this affects the reliability of the test. Conducting the experiment with the least amount of stress to the animals is very important. Also, at the beginning of the study, it is important to ensure that animals are uniformly distributed between diet treatment groups based upon their initial weight at the beginning of the study. Taking accurate weights of the animals at regular intervals, as well as of the feed provided and consumed are necessary for reliable results.

Chemical scoring methods

There has been resistance to a wider adoption of chemical scoring methods for predicting protein quality because of the number of separate assays required. A standard amino acid analysis, plus separate assays for tryptophan and methionine/cysteine, are required. All the critical factors associated with protein hydrolysis, sample preparation for amino acid analysis, proper use of and selection of internal and external standards, selection of analytical method including derivatization and chromatographic method, sensitivity, and possible interference from sample constituents, as well as data acquisition and analysis, will all play roles in determining reliability of using a chemical scoring method for estimating protein quality. Correlation of these results with those for in vivo assays is not guaranteed. Analytical results for lysine may not accurately reflect chemically or biologically available lysine (Friedman, 1996), and so additional testing to predict bioavailability is needed. Protein digestibility is often conducted in conjunction with PDCAAS using an in vitro assay. Again, the critical issue with this method is whether it will accurately reflect how well a protein and its constituent amino acids can be used by an organism for growth and maintenance.

Digestibility methods

High protein digestibility does not necessarily mean high protein quality. Protein quality measures the balance between the amino acids in the protein necessary for growth and cell maintenance.

In vivo tests require animal feeding studies, and all of the factors outlined under PER apply. In addition, for digestibility calculations, accurate measurement of feed consumed, complete recovery of feces (devoid of feed), possibly urine, and calculation of body composition are required. These are not the most pleasant experimental protocols to conduct, but have to be done carefully and correctly if meaningful results are to be obtained.

In vitro protein digestibility requires preparation of a treated sodium caseinate standard. Because of the empirical nature of these determinations, it is critical that results for the so-

dium caseinate are reliable. Preparation of this standard must be conducted exactly as described, and the standard must be tested to ensure that digestibility is 100±2% after it has been prepared. Nitrogen content of the protein standard and the test proteins must be accurately determined using an appropriate Kjeldahl method, since the digestibility assays are calculated on a constant-nitrogen basis. Protein samples must be held so that they do not take up or lose moisture between the time of the N determination and the digestibility assay. For certain protein ingredients, it may be necessary to correct the total N content for nonprotein nitrogen. Preparation of fresh enzyme solutions for each series of assays is critical. It is best that these solutions not be used for more than a couple of hours. Accurate preparation is important, and as the specific activity of each preparation may be somewhat different, the activity should be confirmed before the enzyme is used. Whether a pH shift or a pH stat assay is employed, accurate calibration of pH at the assay temperature is critical. Also, it will be important to select a reaction vessel that can be kept at a constant temperature and be stirred and into which a pH electrode can be inserted for measurement.

Troubleshooting

Protein efficiency ratio and in vivo digestibility methods

Maintaining animals in a comfortable environment, as stress free as possible, is important, including maintaining a constant temperature and uniform photoperiod. If animals appear sick or lethargic, or fail to eat, a veterinarian should be contacted and study terminated. Because some proteins are not highly palatable, conducting a test run, feeding a small number of rats a control diet and some others the experimental diet, is recommended to see how well the animals eat the diet. Sometimes replacing a small amount of the corn starch in the diet (~10%) with dextrose will improve palatability.

Animals should be watered and feed should be replaced at the same time of day, if possible, during the course of the study. Food consumption should be checked daily, and more often if necessary, depending upon feed consumption rate. Animal food dishes should not be left empty.

If animals spill food, the spilled food must be recovered as completely as possible and weighed. If this is a recurrent problem, switch to feed dishes of a different configuration, or alter the bulk density of the food (e.g., switch to a pelleted food, although this is not always possible). Line cages with paper to make collection of spilled feed easier. If paper becomes wet from spilled water or urine, dry the paper at room temperature before recovering and weighing the spilled feed. Spilled feed should be recovered and weighed daily.

The container used to weigh the rats should be large enough so that they cannot jump out. A small wire cage or basket is recommended.

Recovering feces for protein digestibility measurements can be tedious and unpleasant. It is necessary to recover as much material as possible from cage walls and floor, and from the cage liner, with as little contamination with food or urine as possible. Feces from each rat should be collected separately and stored in a sealed container, preferably in the freezer until analyzed. These should be air dried first before storage if possible. If it becomes difficult to recover feces from the cage liner because they stick, switch to a glossier paper to line the cage. In extreme situations, where there is a large amount of urine or water contamination of the feces, measuring nonprotein nitrogen may be necessary to correct for urine contamination (Varner et al., 1953).

Conducting body composition analysis on rat carcasses presents challenges that are usually not faced when taking representative samples, because of hair, bone fragments, etc. Taking additional samples for analysis is recommended, until the standard error for these measurements falls into line with lab averages with more conventional samples.

Measuring Kjeldahl nitrogen is not trivial, particularly on samples with relatively low amounts of nitrogen. Running test proteins with known amounts of nitrogen will help to ensure that determinations are accurate. Titration values should be checked with known standards daily, particularly in the case of samples for which discerning the color change is difficult. Switching catalysts can sometimes help to improve results.

Chemical scoring methods

Amino acid analysis is fraught with literally dozens of pitfalls. For novel protein ingredients, those that have undergone chemical modification, or ones that have been subjected to high heat, conventional methods for protein hydrolysis may or may not be effective. It may be necessary to evaluate different hydrolysis methods to determine which provides the best recovery.

Chromatographic separation of amino acids in the test protein is required for chemical scoring methods. The selectivity and sensitivity of chromatographic methods vary and comparing results of more than one method may be required, particularly when proteins with unusual amino acid profiles are being tested.

For routine regulatory and food labeling purposes, an accurate determination of amino acid content coupled with an assessment of protein provides a sufficient basis for estimating protein quality (Young and Pellet, 1991). However, this does not mean that values are accurate. Unlike animal bioassays, a chemical scoring method cannot factor in the value of a protein as part of a mixed diet. Also, it does not take into account mechanisms animals have for salvaging and reutilizing endogenous amino acids.

In vitro protein digestibility

pH shift and pH stat assays are, in principle, fairly straightforward to conduct. However, it is critical the pH meter be calibrated at 37°C with 37°C buffer. Checking the sensitivity of the meter at the temperature is important.

Also, the effect of stirring on pH fluctuation should be checked for pH stat determinations.

Protein materials that are not highly soluble at pH 8 in water are difficult to test using this method. Hydrating a protein overnight is sometimes helpful.

Check the activity of enzymes separately if digestibility values seem to be low. Ensure that the correct enzyme, grade, and concentration have been used. If there is any question about enzyme quality, replace it.

Anticipated Results

PER

PER values for test proteins will be lower than casein for conventional test materials. This is not the case for protein ingredients that have been specifically designed to have enhanced protein quality. Test results for other high-quality protein sources, such as meat, fish, and egg, may yield higher PER results than a casein control, and the relative rating between very high protein sources is related in part to the palatability of the respective diet. Sometimes the range of weights (weight loss) for animals fed zero-protein diets can be highly variable within the treatment group, reducing the precision of the assay.

Chemical score

Values for a test protein will be lower than for the reference protein. The reliability of results for chemical scoring methods depends upon the accuracy of the amino acid determinations that form the basis for these assays. Chemical scoring and animal bioassays tend to provide similar relative rankings of protein quality; however, the actual values may be different. Chemical scoring methods should not replace a bioassay for testing the quality of a food protein for which there is very little nutritional information.

Digestibility assays

The relative ranking of protein ingredients by in vivo and in vitro digestibility tests will generally track each other; however, the values on a percentage basis may be quite different. The digestibility of a protein will vary depending upon how it has been processed. Proteins that are highly soluble in water, or those that have been treated to improve solubility, will tend to have greater digestibilities. The digestibility values for proteins that have been partially hydrolyzed may not be reliable. For proteins that have been heated, or which have been derivatized (e.g., by formation of browning reaction products or exposure to alkali or acid), in vitro digestibility results may not provide a good indication of the bioavailability.

Time Considerations

PER

The preparation time for a PER is extensive and involves several days for acquisition of rats and their acclimation before a feeding trial begins. Preparation of a consistent and uniform diet is not trivial. Adequate diet for each treatment for the course of the study should be prepared shortly in advance of the study. Testing of the diet involves chemical analysis for protein (i.e., Kjeldahl N takes several hours) and accurate determination of other diet constituents (e.g., ash, crude lipid, and dietary fiber) so that isocaloric diets can be formulated between treatment groups. These determinations take ≥1 day to complete. It is recommended that multiple samples from each diet be obtained for proximate analysis of diet before it is fed to ensure that each diet has the proper nitrogen content (and this is same between diet treatments) and that all diets are isocaloric.

This is a growth assay and takes 28 days to complete. Fecal N and body composition

analyses each take an additional several days of sample preparation and analysis to complete.

Chemical scoring methods

A fairly complicated sample preparation is involved. Comparison of hydrolysis and chromatographic procedures may be necessary when testing unconventional protein sources. Knowing the sample nitrogen content is required. There is also the need to hydrolyze protein (an overnight procedure). Separate hydrolysis procedures are required for tryptophan and for the sulfur-containing amino acids, which take several hours (or overnight) to complete. Chromatographic analysis is relatively rapid (minutes to hours); however, becoming proficient at amino acid chromotography can take months or years.

Digestibility tests

In vitro assays themselves take a few hours to complete. The protein nitrogen content of a sample must be known. Sample hydration can take one to several hours. Preparation of enzyme solutions takes <1 hr, however, determination of the activity of the different enzymes within the preparation can take days. In vivo digestibility assays involve a feeding study, and the investigator faces similar issues as described for PER.

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Evaluation of the Progress of Protein Hydrolysis

This unit describes several techniques that can be used to evaluate the progress of protein hydrolysis by using the concept of degree of hydrolysis (DH). The DH is defined as the percentage of hydrolyzed peptide bonds. The techniques described here are based on the assumption that a free amino group and a free carboxyl group are released every time a peptide bond is hydrolyzed. They quantify the increase in the concentration of such groups as a variable to evaluate the progress of hydrolysis.

The ninhydrin reaction (see Basic Protocol 1), the TNBS reaction (see Alternate Protocol 1), the fluorescamine reaction (see Alternate Protocol 2), and formol titration (see Alternate Protocol 3) all evaluate released amino groups by comparing the amounts of free amino groups before and after hydrolysis. The first three methods are spectrophotometric techniques, whereas the fourth is a potentiometric technique. The first and second are chromogenic techniques, whereas the third is fluorometric. These techniques are usually performed as time-course experiments. As the hydrolysis reaction proceeds, aliquots (samples) of the reaction are taken periodically and treated with a test reagent. Products of this reaction are proportional to the amount of free amino groups at each time point.

One additional technique presented is the pH-stat technique (see Basic Protocol 2). This is a kinetic technique used to evaluate the progress of hydrolysis. The hydrolysis reaction is followed in the same reaction vessel at theoretically infinitesimal increments. This gives a graph of the progress of the hydrolysis. The pH-stat technique is useful for evaluating the progress of an enzyme-catalyzed protein hydrolysis process on an industrial scale, where the degree of hydrolysis is the most important variable in ensuring that products with desirable functional and organoleptic properties are obtained.

STRATEGIC PLANNING

Protein hydrolysates are usually produced by limited enzymatic hydrolysis of protein molecules in foodstuff, yielding polypeptides that are smaller in molecular mass. Protein hydrolysis has several aims. The most common is to make the protein moiety of a foodstuff soluble by reducing the size of the peptides. Solubilization simplifies isolation of the protein moiety by physical means. Protein hydrolysis has also been applied to improve the functional, organoleptic, and nutritional value of a foodstuff. Advances in the technology of protein hydrolysate production has allowed the use of unconventional protein sources for animal and human food.

The following test parameters must be defined for the production of a protein hydrolysate: the mass of the hydrolysis mixture (M in g or kg), the substrate (S in % w/w) where protein concentration ($N \times f_N$) is determined by the Kjeldahl method (*UNIT B1.2*), the enzyme/substrate ratio (E/S, as activity units per gram or kilogram of substrate), the pH, and the temperature (°C). Also, the conditions needed to terminate hydrolysis must be defined. Because hydrolysis is typically allowed to proceed until a given degree of hydrolysis (DH) value is reached, the reaction is terminated by a sudden inactivation of the enzyme. Sometimes, termination is achieved by changing the pH. If a reaction is performed under alkaline conditions, lowering the pH to 4.0 to 4.5 concludes hydrolysis. For thermosensitive enzymes, rapidly heating the reaction mixture is suitable. In both cases, preliminary assays should be performed to determine satisfactory conditions, especially to determine the appropriate time at which to inactivate the enzyme under real reaction conditions.

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When producing protein hydrolysates, the next step is often to separate the solubilized protein from the nonhydrolyzed material. Centrifugation or filtration yields soluble protein and a precipitate or sludge.

BASIC PROTOCOL 1

NINHYDRIN REACTION TO DETERMINE DEGREE OF PROTEIN HYDROLYSIS

In the following protocol, amino acids are reacted with ninhydrin hydrate at pH 5 and 100°C for a standard period of time, yielding a purple-blue compound, the ammonium salt of diketohydrindylidene-diketohydrindamine, as the major product. The structure of the reagents and the reaction is shown in Figure B2.2.1. Amino acids generally react with ninhydrin to yield carbon dioxide, ammonia, and, usually, an aldehyde of one carbon atom fewer than the original amino acid. Ninhydrin also reacts with ammonia and primary amines. The absorbance of the purple-blue product is measured at 570 nm. Proline and hydroxyproline give a yellow product whose absorbance is measured at 440 nm (Fig. B2.2.2).

NOTE: A variety of assay conditions can be used here; this protocol is specific for Alcalase. Many other enzymes can be used, as well as other percentages of protein and other enzyme/substrate ratios.

Materials

Substrate: sample solution containing native protein
Enzyme (e.g., Alcalase 0.6L or Alcalase 2.4L; Novo Nordisk Biochem), adjusted to desired reaction pH using HCl or NaOH
Water or buffer at desired pH and temperature
3 N HCl
Standard solution:1.5 mM leucine (1.5 meq NH_2/g) in 50% (v/v) isopropanol
Standard solution: 1.5 mM proline or hydroxyproline (1.5 meq NH_2/g) in 50% (v/v) isopropanol
Ninhydrin reagent (see recipe)

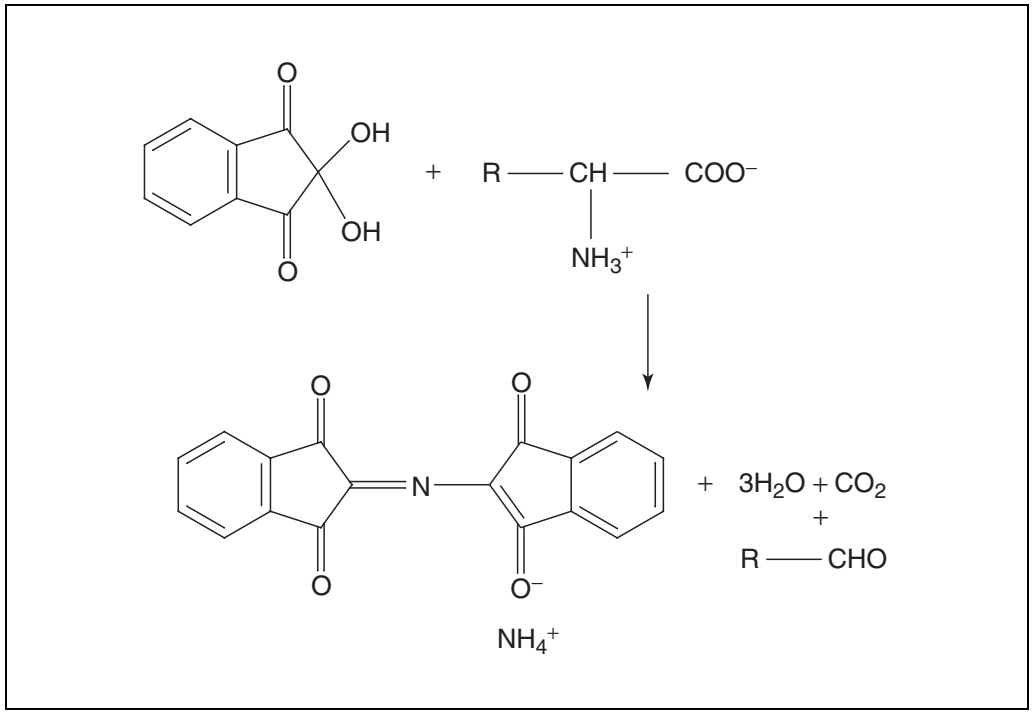

Figure B2.2.1 Reaction of ninhydrin with an amino acid, producing the purple-blue product.

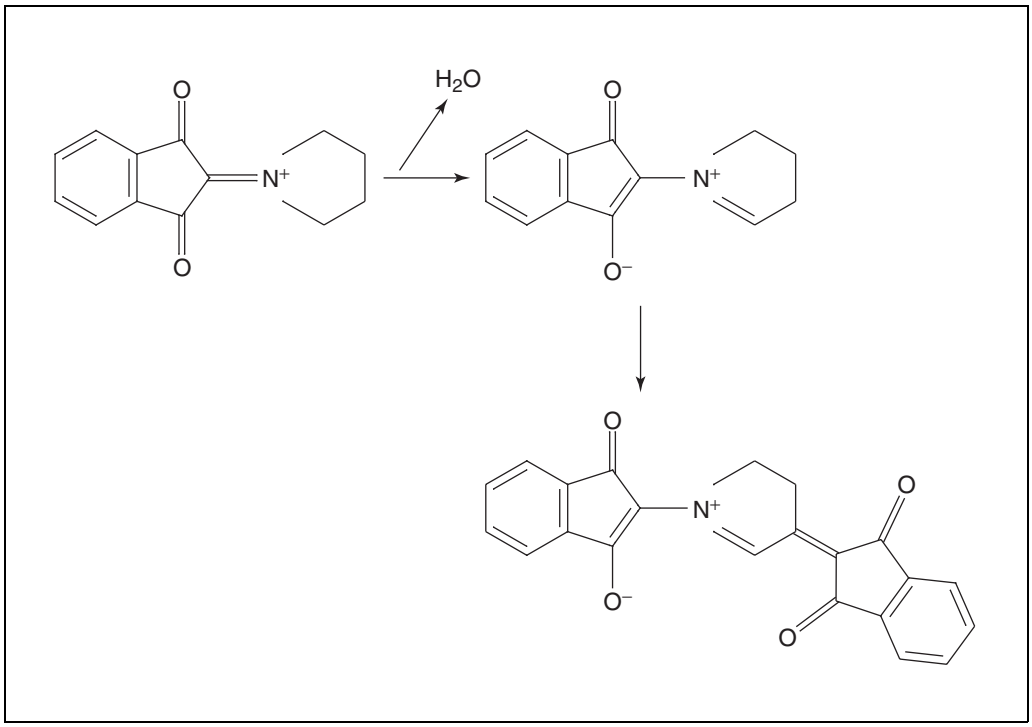

Figure B2.2.2 Reaction of ninhydrin with proline or hydroxyproline, producing the yellow product.

Water baths at 85° (optional) and 100°C
13 × 100–mm glass test tubes
Spectrophotometer (visible range, 440 to 570 nm)
Glass spectrophotometer cuvettes

Prepare hydrolysis samples

1. Determine the appropriate amounts of substrate and enzyme to use for the hydrolysis reaction. Use an amount of substrate that gives protein at 8% (w/w) of the total reaction and calculate enzyme at a ratio of 0.012 Anson U/g protein. For example, for a 100-ml Alcalase hydrolysis reaction, use:

   substrate containing 8 g protein
   0.16 g Alcalase 0.6L or 0.04 g Alcalase 2.4L
   buffer to 100 ml.

   The amount of protein in the substrate solution can be determined by using the Kjeldahl method (UNIT B1.2) to determine the nitrogen concentration (N) and then multiplying this by a factor of 6.25 (f_N).

   The mass of enzyme in grams (M_E) is calculated $M_E = M_P \times (A/E)$, where (M_P) is the mass (in g) of protein in the substrate, A is the suggested enzyme activity in Anson units/gram (0.012 AU/g), and E is the actual enzyme activity specified by the manufacturer. For Alcalase, 0.6L and 2.4L correspond to 0.6 and 2.4 AU/g, respectively. Thus M_E = 8 g × (0.012 AU/g ÷ 0.6 AU/g) = 0.16 g for 0.6L, or 0.04 g for 2.4L.

2. Mix substrate in water or an appropriate buffer at the desired temperature and pH.

   Every sample should be analyzed with appropriate replicates.

3. Before adding enzyme, transfer a 1.0-ml sample to a 13 × 100–mm glass test tube to be used for determining free amino groups in the raw material.

4. Add enzyme to the remaining substrate solution and stir mixture throughout the course of the reaction.

5. Take 1.0-ml samples at suitable time intervals during the reaction and transfer to glass test tubes. Immediately inactivate each sample by lowering the pH value to 4.0 with 3 N HCl (10 to 20 µl) or by transferring the sample to a water bath at 85°C for at least 15 min.

   If HCl is used to stop the reaction, it should also be added to the control (step 3) and to the standards and blank (steps 6 and 7) to maintain consistent dilution of amino equivalents.

Perform ninhydrin reaction

6. Prepare a series of 1-ml calibration standards by diluting 1.5 mM leucine standard solution containing 0.01 to 0.15 amino meq/g in the same solvent that was used to prepare the sample protein. Prepare a series of proline or hydroxyproline standards in the same manner.

   As with the samples, the standards and blank should be prepared in appropriate replicates.

7. Prepare a blank containing 1 ml solvent alone.
8. Add 0.2 ml ninhydrin reagent to each tube (steps 3, 5, 6, and 7).
9. Heat 10 min at 100°C and cool to room temperature.

Analyze

10. Measure absorbance at 570 nm for any amino acid, and at 440 nm for proline and hydroxyproline.
11. Create separate calibration curves for leucine and proline by plotting A_{570} or A_{440}, respectively, versus equivalent amino acid standard concentration.
12. For each time point, determine the concentration equivalents of amino groups in the samples by extrapolating from each standard curve.
13. For each time point, separately determine the amount of released amino groups (h) by subtracting the value of the corresponding unhydrolyzed control (step 3) in the corresponding standard curve.

    This gives the number of equivalents of peptide bonds hydrolyzed (h), expressed as meq/g protein from the following equation:

    $h = (A \times b)/m$

    where A is the absorbance at 570 or 440 nm and b and m are the y intercept and slope of the calibration curve. To get h, sum the values of h from 570 and 440.

14. Determine the degree of hydrolysis at each time point from the following equation:

    $DH = h/h_{tot} \times 100\%$

    where h_{tot} is the total amount of peptide bonds.

    When h_{tot} remains unknown, 8 amino meq/g protein is a good estimate (Adler- Nissen, 1986).

ALTERNATE PROTOCOL 1

TNBS REACTION TO DETERMINE DEGREE OF HYDROLYSIS

Trinitrobenzenesulfonic acid hydrate (TNBS) reacts with amino acids, yielding a yellow product (Fig. B2.2.3) whose absorbance is measured at 340 nm. TNBS reacts only with amino groups in their unprotonated state. TNBS binds to amino acids in two steps: (1) a fast reaction with low affinity, and (2) a slow reaction with relatively high affinity. For practical purposes, the two-step reaction should be considered as one. TNBS reacts with primary amines under slightly alkaline conditions, and lowering the pH stops the reaction.

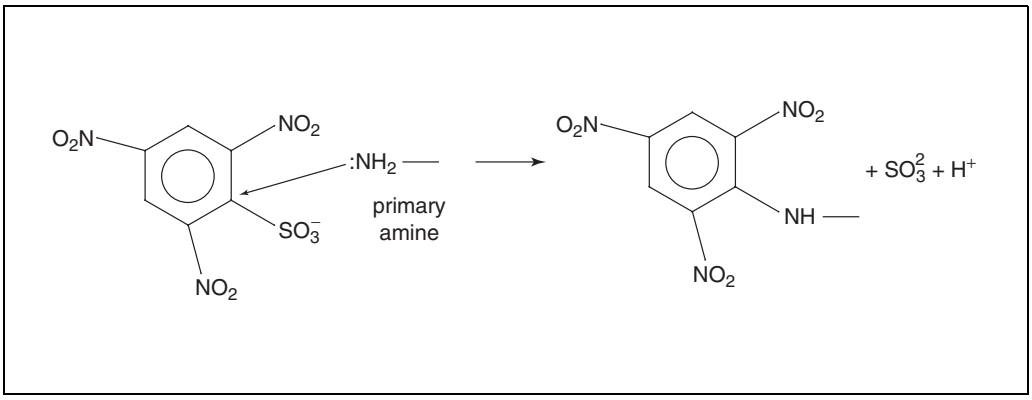

Figure B2.2.3 TNBS reaction.

Additional Materials *(also see Basic Protocol 1)*

1% (v/v) SDS (*APPENDIX 2A*)
0.2 M sodium phosphate buffer, pH 8.2 (*APPENDIX 2A*)
2.5 mM leucine standard in 1% SDS
0.1% (w/v) trinitrobenzenesulfonic acid dihydrate (TNBS) in deionized water (prepare immediately before use and protect from light)
0.1 N HCl

15 × 85–mm and 16 × 125–mm glass test tubes
50° and 75°C shaking water baths
Spectrophotometer (visible range; 340 nm)

Prepare hydrolysis samples

1. Determine the appropriate amounts of substrate and enzyme to use for the hydrolysis reaction. Use an amount of substrate that gives protein at 8% (w/w) of the total reaction and calculate enzyme at a ratio of 0.012 Anson U/g protein. For example, for a 100-ml Alcalase hydrolysis reaction, use:

   substrate containing 8 g protein
   0.16 g Alcalase 0.6L or 0.04 g Alcalase 2.4L
   buffer to 100 ml.

   The amount of protein in the substrate solution can be determined by using the Kjeldahl method (UNIT B1.2) to determine the nitrogen concentration (N) and then multiplying this by a factor of 6.25 (f_N).

   The mass of enzyme in frams (M_E) is caluculated $M_E = M_P \times (A/E)$, where (M_P) is the mass (in g) of protein in the substrate, A is the suggested enzyme activity in Anson units/gram (0.012 AU/g), and E is the actual enzyme activity specified by the manufacturer. For Alcalase, 0.6L and 2.4L correspond to 0.6 and 2.4 AU/g, respectively. Thus M_E = 8 g × (0.012 AU/g ÷ 0.6 AU/g) = 0.16 g for 0.6L, or 0.04 g for 2.4L.

2. Mix substrate and appropriate buffer at the desired temperature and pH.

   Every sample should be analyzed with appropriate replicates.

3. Before adding enzyme, take a 0.25-ml sample and transfer to a 15 × 85–mm glass test tube containing 2 ml of 1% SDS and incubate 15 min at 75°C.

   This unhydrolyzed control will be used for evaluation of free amino groups in the raw material.

4. Add enzyme to the remaining substrate solution and stir mixture throughout the course of the reaction.

5. Take 0.25-ml samples at suitable time intervals during the reaction. Immediately transfer each sample to a test tube containing 2 ml of 1% SDS and keep at 75°C in a shaking water bath for 15 min.

   This inactivates the enzyme and disperses the protein hydrolysate.

Perform TNBS reaction

6. Transfer 0.25 ml from each sample (step 5) and control (step 3) to separate 16 × 125–mm glass test tubes containing 2 ml of 0.2 M sodium phosphate buffer.
7. Prepare six to ten calibration standards by diluting 2.5 mM leucine standard containing 0.25 to 2.5 amino meq/g in sodium phosphate buffer at a total volume of 2.25 ml.

   As with the samples, the standards and blank should be prepared in appropriate replicates.

8. Prepare a blank containing 0.25 ml of 1% SDS in 2 ml sodium phosphate buffer.
9. Add 2 ml of 0.1% TNBS solution to each tube, vortex, and incubate in the dark at 50°C for 60 min.
10. Stop the reaction by adding 4.0 ml of 0.1 N HCl. Allow samples to reach room temperature and read absorbance at 340 nm.

Analyze

11. Create a calibration curve by plotting A_{340} versus concentration of the standards.
12. Determine the degree of hydrolysis (see Basic Protocol 1, steps 12 to 14).

ALTERNATE PROTOCOL 2

FLUORESCAMINE REACTION TO DETERMINE DEGREE OF HYDROLYSIS

Fluorescamine reacts with primary amines to form fluorophores (see Fig. B2.2.4) that are excited at 390 nm and fluoresce at 475 nm. Peptides react with fluorescamine at pH 7.0, giving higher fluorescence than amino acids, which have maximum fluorescence at pH 9. The reaction proceeds rapidly with primary amines at 25°C. The resulting fluorescence is proportional to the amine concentration. The fluorophores are stable for several hours. A negligible interference is produced with ammonia.

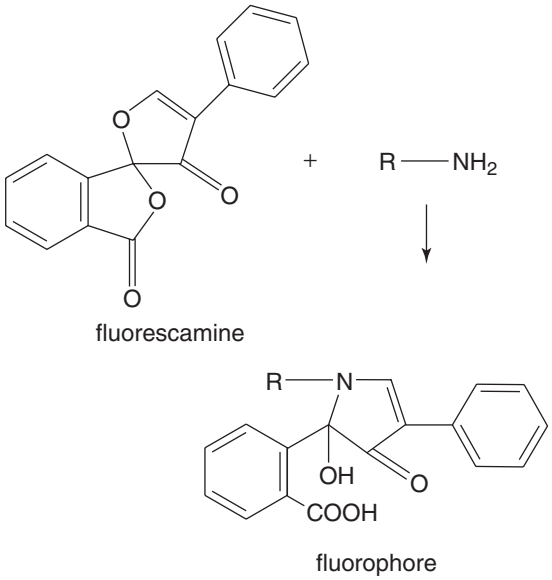

Figure B2.2.4 Fluorescamine reaction.

Additional Materials *(also see Basic Protocol 1)*

0.04 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*) *or* 0.04 M borate buffer, pH 9.7 (see recipe)
0.3 mg/ml fluorescamine reagent in acetone
Spectrofluorometer (excitation 390 nm; emission 475 nm)

1. Perform enzymatic hydrolysis of protein samples at a series of time points (see Basic Protocol 1, steps 1 to 5). Prepare a series of standards and a blank (see Basic Protocol 1, steps 6 and 7).
2. Set the spectrofluorometer to 390 nm excitation and 475 nm emission.
3. To a glass spectrofluorometer cuvette, add 0.20 ml of 0.04 M sodium phosphate buffer, pH 7 (for peptides) or borate buffer, pH 9.7 (for free amino acids).

   Usually, in food technology, the hydrolysis of a protein product is not intended to proceed to the production of free amino acids, because extended hydrolysis yields a product with poor functional properties. Therefore, it is important to know the amount of free amino acids generated by the hydrolysis. The TNBS reaction (see Alternate Protocol 1) allows a useful tool to quantify the amount of free amino acids.
4. Add 0.20 ml of 0.3 mg/ml fluorescamine and 1.0 ml of hydrolysis sample containing 5 to 50 nmol amino acid.
5. Measure fluorescence of the mixture at 475 nm.

   The resulting fluorescence is proportional to the amine concentration.
6. Create a calibration curve by plotting fluorescence versus concentration of the standards.
7. Determine the degree of hydrolysis (see Basic Protocol 1, steps 12 to 14).

FORMOL TITRATION TO DETERMINE DEGREE OF HYDROLYSIS

ALTERNATE PROTOCOL 3

Amino acids react with formaldehyde, liberating one H^+ ion from the amino group, which is potentiometrically titrated with a sodium hydroxide solution. (Formol titration is also useful for measuring total free amino acids; for titration of acid tastants, see *UNIT G2.1*.) Amino groups of histidine do not react and those of proline and hydroxyproline react about 75%. Tertiary amines such as those in the guanidine group do not interfere with the reaction. Protein containing significant amount of Pro and hydroxyPro should be evaluated using other techniques.

$$H_2C{=}O + H_2N{-}CRH{-}COOH \rightarrow HC{=}N{-}CRH{-}COOH + H^+ + H_2O$$

Additional Materials *(also see Basic Protocol 1)*

0.25 N NaOH
35% (w/v) formaldehyde, pH 8.1 (adjust pH with 10 M NaOH)

13 × 100–mm glass test tubes
10-ml buret or potentiometric titration apparatus

1. Perform enzymatic hydrolysis of protein samples at a series of time points (see Basic Protocol, steps 1 to 5) and prepare a sequence of standards and a blank (see Basic Protocol 1, steps 6 and 7). Scale up all samples, standards, and blanks to 2.5 ml in 13 × 100–mm glass test tubes.
2. Adjust pH in all tubes to 8.1 with 0.25 N NaOH.

Table B2.2.1 Calibration Factors (1/α) for pH-Stat at Various Temperatures[a]

Calibration factor					
pH	Temperature (pK for amino groups)				
	25°C (7.7)	30°C (7.6)	40°C (7.3)	50°C (7.1)	60°C (6.9)
6.5	—	—	—	5.00	3.50
7.0	—	5.00	3.00	2.27	1.79
7.5	2.59	2.27	1.63	1.40	1.25
8.0	1.50	1.40	1.20	1.13	1.08
8.5	1.16	1.13	1.06	1.04	1.03
9.0	1.05	1.04	1.02	1.01	1.01
9.5	1.02	1.01	1.01	1.00	1.00
10.0	1.01	1.00	1.00	1.00	1.00
10.5	1.00	1.00	1.00	1.00	1.00
11.0	1.00	1.00	1.00	1.00	1.00

[a]From Adler-Nissen (1986) with permission.

3. Add 1 ml of 35% formaldehyde, pH 8.1.
4. Incubate 1 min at room temperature and titrate with 0.25 N NaOH to pH 8.1. Record the volume of NaOH needed to reach the potentiometric endpoint.

   If more than 2 ml of 0.25 N NaOH is needed, repeat the assay using 1.5 ml formaldehyde.
5. Calculate the degree of hydrolysis (DH) of the substrate from the equation:

   $$DH = B \times N_B \times 1.5 \times (1/M_P) \times (1/h_{tot}) \times 100\%$$

   where B is the volume of NaOH used for maintain the pH at 8.1 value, N_B is the normality of the base, 1.5 is 1/α (Table B2.2.1), and h_{tot} is 8.2 (for casein; Table B2.2.2).

Table B2.2.2 Content of Peptide Bonds for Various Food Proteins[a]

Protein material source	h_{tot} in meq/g ($N \times f_N$)
Casein	8.2
Whey protein concentrate	8.8
Meat	7.6
Hemoglobin	8.3
Gelatin	11.1
Fish protein concentrate	8.6
Soya proteins	7.8
Wheat gluten	8.3
Maize protein isolate	9.2

[a]From Adler-Nissen (1986) with permission.

PROTEIN HYDROLYSIS USING THE pH-STAT TECHNIQUE

The pH-stat technique monitors the course of a reaction in which each peptide bond is hydrolyzed by one or several enzymes. pH-stat evaluates the progress of hydrolysis by titrating the released amino groups with an alkaline solution. Enzymes work at constant pH and temperature during the entire process, so that no buffering is needed.

An automated pH-stat method gives a direct measurement of the percentage of hydrolyzed peptide bonds, the degree of hydrolysis (DH). The DH is calculated using the equation:

$$DH = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100\%$$

where B (ml) is the volume of base consumed, N_B is the normality of the base, $1/\alpha$ is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at particular pH and temperature (Table B2.2.1), M_P (g) is the amount of the protein in the reaction mixture, and h_{tot} (meq/g) is the sum of the millimoles of individual amino acids per gram of protein associated with the source of protein used in the experiment (Table B2.2.2; Adler-Nissen, 1986).

As an example, this protocol describes how to perform the reaction using a casein substrate and a commercial proteolytic enzyme cocktail. The activity of the commercial four-enzyme cocktail is 35 U/ml, but should be tested prior to the experiment in order to determine the appropriate amount of enzyme needed for the reaction. The proteolytic activity assay (García-Carreño et al., 1994) is described below.

The pH-stat method can be modified and applied to a particular enzyme or substrate to assay any enzyme/substrate combination. The substrate can come from any source of protein such as poultry, milk, soybean, or fish processing byproducts. The amount of protein in the reaction should not exceed 8% (as calculated in Basic Protocol 1). The enzyme can be any alkaline endopeptidase such as Alcalase, trypsin, or chymotrypsin, and should be used in the proportions indicated in Basic Protocol 1. The selection of the appropriate enzyme depends on its efficiency and cost.

Materials

Four-enzyme cocktail (see recipe)
50 mM Tris·Cl, pH 8.0 at 25°C (*APPENDIX 2A*)
1% (w/v) azocasein in 50 mM Tris·Cl, pH 8.0 at 25°C
20% (w/v) trichloroacetic acid (TCA)
0.1 N NaOH
Casein from bovine milk powder, purified (Sigma)
Nitrogen

Spectrophotometer (366 nm)
Thermoregulable reaction vessel fitted with pH meter and temperature sensor
Water jacket or water bath set at 25°C
Autotitrator with 20-ml autoburet and recorder

Determine specific activity of enzyme cocktail

1. In six separate microcentrifuge tubes, mix 7 µl four-enzyme cocktail with 0.5 ml of 50 mM Tris·Cl, pH 8.0.

   A sample and a blank are each assayed in triplicate.

2. Add 0.5 ml of 20% TCA to the blank tubes only.

   For the blanks, 20% TCA is added before the substrate to inactivate the enzyme.

3. Initiate the reaction by adding 0.5 ml of 1% azocasein to all tubes.

4. Incubate 10 min and stop the reaction in the sample tubes by adding 0.5 ml of 20% TCA.
5. Microcentrifuge the reaction mixture 5 min at 6500 × *g*.
6. Record the absorbance at 366 nm (A_{366}).
7. Average the triplicate sample and blank values and subtract the mean of the blank from the mean of the sample.
8. Calculate the specific activity of the enzyme solution:

   activity (U/ml) = A_{366}/time (min)/volume (ml)

   For the four-enzyme cocktail used in this protocol, García-Carreño et al. (1994) reported an A_{366} of 0.49, giving an activity of 0.49/10 min/0.007 ml = 7 U/ml. The activity may vary depending on the stability of the enzymes under the given storage conditions.

Perform pH-stat assay

9. Calculate the total amount of raw substrate material (M_R) needed for the reaction:

   $M_P = M \times S\%$

   $M_R = M_P/P\%$

   In the first equation, M is the total mass of the hydrolysis reaction (10 g) and S% is the desired percent of substrate protein (casein) in the reaction (1%), giving the desired mass of substrate protein (M_P = 0.1 g). In the second equation, P% is the percent of substrate protein in the raw material, equal to the nitrogen content (N, determined by the Kjeldahl method; UNIT B1.2) times a conversion factor (f_N = 6.24). For casein with an N of 14.3%, M_R = 0.1 g/(14.3% × 6.24) = 0.11 g.

10. Calculate the amount of enzyme solution (V_{ES}) needed for the reaction.

    $M_E = E/S \times M_P = 2\% \times 0.1$ g = 2 mg

    $V_{ES} = M_E$/[protein] = 2 mg/(2.8 mg/ml) = 0.71 ml

    The mass of enzyme (M_E) is based on a recommended enzyme/substrate ratio of 2%. The four-enzyme cocktail (see Reagents and Solutions) has 2.8 mg protein/ml, so a volume of 0.71 ml is needed. At 7 U/ml, this gives 5 U enzyme in the reaction.

11. Calculate the amount of water (M_W) needed for the reaction:

    $M_W = M - M_{ES} - M_R$ = 10 g − 0.71 g − 0.11 g = 9.18 g = 9.18 ml

12. Adjust the pH of substrate and enzyme solutions to 8.0 using 0.1 N NaOH.
13. Add 9.29 ml of casein substrate solution (total substrate and water calculated in steps 9 and 11, respectively) to a thermoregulable reaction vessel, insert pH and temperature sensors, and stir the solution using a magnetic stirrer. Set up an autotitrator with a 20-ml autoburet.

    Be careful not to stir too fast, as excess agitation can introduce CO_2 into the solution and change the pH.

14. Set the pH meter to 8.0, set the temperature sensor to 25°C, and charge the autoburet with 0.1 N NaOH solution.
15. Once the pH is constant, pump nitrogen over the reaction mixture.
16. Record the actual pH (should be 8.0).
17. Add 0.71 ml enzyme solution (total 10 ml reaction) and start the automatic titration.

The pH value of the reaction mixture goes down due to proteolytic activity, and is instantaneously maintained at 8.0 by the addition of NaOH solution.

18. Monitor titration by recording the volume of NaOH added every 5 min for 60 min or until the desired DH is reached.

19. Plot the progress of hydrolysis as a function of volume (in ml) of NaOH consumed versus time.

20. Calculate the degree of hydrolysis of the substrate from the equation:

$$DH = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100\%$$

where B (ml) is the volume of NaOH used to maintain pH at 8.0, N_B is the normality of the base, $1/\alpha$ is 1.5 (Table B2.2.1), and h_{tot} is 8.2 (Table B2.2.2).

21. Titrate a blank (same substrate with no enzyme) in the same manner.

No titrant solution should be consumed. Some substrates, while solubilizing, will reduce the pH of the solution and hence cause some consumption to occur; take this amount as zero.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Borate buffer, 0.04 M, pH 9.7

Dissolve 7.624 g sodium tetraborate decahydrate (reagent grade) in 400 ml deionized water. Adjust pH to 9.7 with 1 N NaOH. Bring to 500 ml with water. Store up 3 months at 4°C.

Citrate buffer, 200 mM

Dissolve 21 g citric acid monohydrate (reagent grade) in 400 ml water. Adjust pH to 5.0 with 1 N NaOH. Bring to 500 ml with water. Store up to 3 months at 4°C.

Four-enzyme cocktail

1.6 mg trypsin from porcine pancreas
3.1 mg chymotrypsin from bovine pancreas
1.3 mg aminopeptidase from porcine intestinal mucosa
8.0 mg pronase type XIV from *Streptomyces griceus*
Bring to 5 ml with distilled water
Store in 720–µl aliquots up to 2 months at −20°C

The final protein content of the solution is 2.8 mg/ml. The solution has 7 U/ml of specific activity, calculated using azocasein as substrate (see Basic Protocol 2, steps 1 to 8). All enzymes are available from Sigma.

Ninhydrin reagent

Dissolve 0.8 g $SnCl_2 \cdot 2H_2O$ in 500 ml 200 mM citrate buffer (see recipe) to give a 7 mM $SnCl_2$ solution. Dissolve 20 g crystalline ninhydrin in 500 ml 2-methoxyethanol (methyl cellosolve, Brenntag) to give a 4% (w/v) solution. Combine 500 ml $SnCl_2$ solution and 500 ml ninhydrin solution. Purge with nitrogen and store in dark bottles for up to one week at 4°C.

CAUTION: *Methyl cellosolve is toxic and ninhydrin stains everything it contacts.*

COMMENTARY

Background Information

Proteins are one of the most important ingredients in food production for both animals and humans. Besides having nutritional properties, protein contributes to the functional and organoleptic properties of food. The nutritional value of a protein depends on the total essential amino acid content. However, the availability of amino acids is conditioned by some protein attributes, mainly digestibility.

The most important feature affecting the functional and organoleptic properties of a protein is its surface structure. Surface structures affect the interaction of a protein with water or other proteins. By modifying the structure of the protein, particular functional and organoleptic properties are obtained. Functional properties of a protein are physicochemical characteristics that affect the processing and behavior of protein in food systems (Kinsella, 1976). These properties are related to the appearance, taste, texture, and nutritional value of a food system. Hydrolysis is one of the most important protein structure modification processes in the food industry. Proteins are hydrolyzed to a limited extent and in a controlled manner to improve the functional properties of a foodstuff.

In any quantitative work on protein hydrolysis, it is necessary to have a measure of the extent of the hydrolytic degradation. The measurement of the number of peptide bonds cleaved during a hydrolytic process is related to the activity of proteinolytic enzymes and the extent of hydrolysis. Various techniques that evaluate the progress of hydrolysis have been reported, such as the trichloroacetic acid (TCA) solubility index, which evaluates the percentage of nitrogen soluble in TCA after partial hydrolysis of the protein.

Degree of hydrolysis

A foodstuff (or other sample) obtained by hydrolysis of a protein material is called a *protein hydrolysate*. The *degree of hydrolysis* measures the percentage of peptide bonds hydrolyzed during protein hydrolysis (Adler-Nissen, 1976). An advantage of the DH concept is that for a given enzyme/substrate system the DH is independent of five variables: substrate concentration, enzyme/substrate ratio, pH, temperature, and time (Adler-Nissen, 1982).

The susceptibility of food protein to hydrolysis by digestive enzymes is called *digestibility* (*UNIT B2.1*). Proteins are hydrolyzed in industry by chemical or enzymatic means, just as they are in the digestive system. Chemical techniques for protein hydrolysis are harsh and extensive, and are generally only useful for amino acid composition analysis. Enzymatic proteolysis is highly specific in both the reactions it catalyzes and the products it yields. Enzymatic reactions can occur under mild conditions of temperature, pH, ionic strength, and pressure. In contrast to chemical hydrolysis, the nutritional value of the amino acids is maintained and functional properties are improved.

The degree of hydrolysis is calculated using hydrolysis equivalents (h), the number of peptide bonds cleaved during hydrolysis, expressed as eq/kg protein or meq/g protein. Hydrolysis equivalents are assayed by measuring the increase in free amino or free carboxyl groups that are produced by the hydrolysis of some protein. Several techniques have been described to assess the amount of amino and carboxyl groups before and after protein hydrolysis. The ninhydrin, TNBS, and fluorescamine reactions and the formol titration technique are used to evaluate the free amino group. The pH-stat technique is used to evaluate the free α-amino groups.

Sampling techniques

The ninhydrin, TNBS, and fluorescamine reactions, and formol titration are usually used to evaluate the time course of protein hydrolysis by periodically taking samples. The hydrolysis product (i.e., the free amino group) in each timed sample is treated with a chromogen or a fluorescent reagent and the product is evaluated by spectrophotometry. The result is proportional to the concentration of free amino groups. In the case of formol titration, reaction of the sample with formaldehyde liberates one H^+ ion from the amino group, which is potentiometrically titrated with a sodium hydroxide solution. Quantification of the number of amino groups gives the number of peptide bonds hydrolyzed and, hence, yields the hydrolysis equivalents, h. For time-course techniques, the DH is related to h by the equation $DH = (h/h_{tot}) \times 100\%$, where h_{tot} is the sum of the millimoles of individual amino acids per gram in the unhydrolyzed protein (Adler-Nissen, 1986).

Several assay techniques are available for quantitating amino groups. The ninhydrin technique is designated as Basic Protocol 1 because it is the most frequently used technique. The

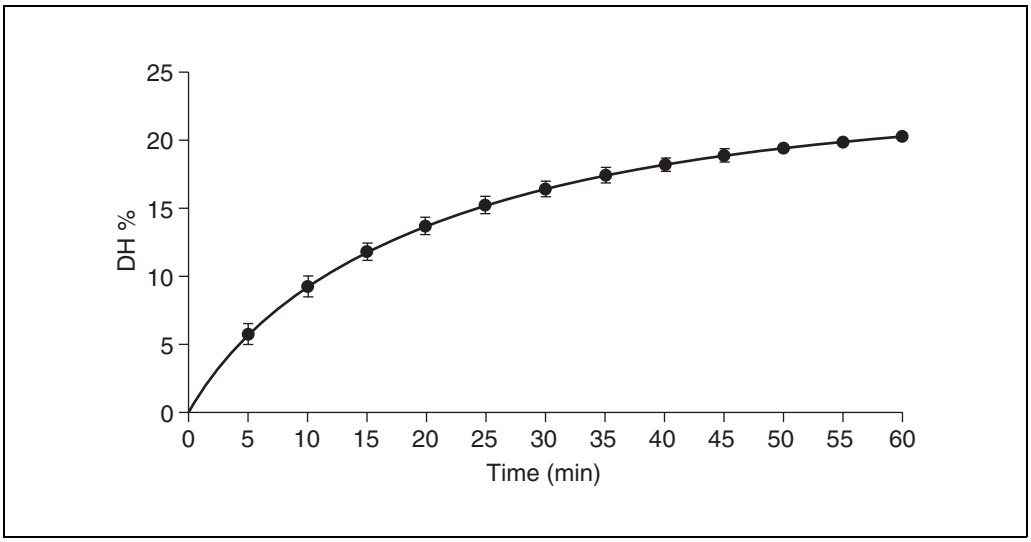

Figure B2.2.5 A typical graph of the progress of protein hydrolysis by the pH-stat technique.

trinitrobenzenesulfonic acid (TNBS) technique was accepted by many researchers for determining the DH of food protein hydrolysates because of its reproducibility. Nevertheless, it was difficult to apply to all food proteins, as some food proteins are difficult to disperse (e.g., partially insoluble proteins). Adler-Nissen (1979) modified this method by dispersing the protein in buffer containing SDS, using a less alkaline phosphate buffer instead of carbonates, and setting the reaction time to 1 hr. This version is presented in Alternate Protocol 1. The fluoroscamine technique (Alternate Protocol 2) is reliable and rapid to evaluate. Formol titration (Alternate Protocol 3) is an alternative when the laboratory does not have pH-stat equipment or a spectrophometer and spectrofluorometer. Comparison of results from more than one of the amino-detecting time-course assays allows for greater confidence.

Kinetic techniques

The pH-stat technique is a strategy used to evaluate the hydrolysis of protein by specific enzymes in a nonbuffered aqueous medium at alkaline pH. Each protein is composed of a specific number of amino acids, h_{tot}, which is expressed as equivalents of peptide bonds per kilogram (or milliequivalents per gram) of protein. The h_{tot} for different food proteins is given in Table B2.2.2.

In some experiments, specific information is needed about the equivalent of peptide bonds hydrolyzed during the reaction, in which case $h = (B \times 1/\alpha \times N_B)/M_P$, where α is the average degree of dissociation of the α-amino groups liberated during the hydrolysis, as determined by the equation $\alpha = (10^{pH-pK})/(1 + 10^{pH-pK})$. It is important to remember that the pK is dependent on temperature. The value of $1/\alpha$ is the calibration factor of α for pH-stat and is shown in Table B2.2.1.

If necessary, the size of peptides generated during the experiment can be evaluated using a 10% to 18% gradient SDS-polyacrylamide gel (Laemmli, 1970; *UNIT B3.1*). It is very important to be sure that there is no residual enzymatic activity when the desired DH has been reached. To guarantee no residual enzyme activity, a useful technique is substrate SDS-PAGE (García-Carreño, 1993).

Critical Parameters and Troubleshooting

The ninhydrin method is affected by sucrose concentrations >10% in samples hydrolyzed with 6 N HCl. The entire procedure is carried out in disposable tubes. Substances such as glycerol, phenol, and mannitol do not affect the assay results.

Anticipated Results

A typical graph of the progress of protein hydrolysis by the pH-stat technique is shown in Figure B2.2.5. The pH-stat technique offers reliability, reproducibility, and simplicity. In addition, the technique has the advantage that no secondary reaction is needed. However, if the equipment needed to perform a pH-stat experiment is not available, the ninhydrin and TNBS techniques are good alternatives.

The progress of protein hydrolysis is currently evaluated by measuring the degree of hydrolysis at intervals. Because a limited controlled hydrolysis of a food protein yields products of particularly interesting functional and organoleptic properties, the DH concept simplifies the evaluation of the progress of the hydrolysis.

Regardless of which technique is used to measure the DH, at least five independent indices can be defined for any given enzyme/substrate system: substrate concentration, *E*/*S* ratio, pH, temperature, and time. The advantage of using the DH is that four of these variables (*S*, *E*/*S*, *T*, *t*) can be replaced by DH (for details, see Adler-Nissen, 1982).

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This book presents the fundamental theory and practice used in biochemistry and food science laboratories.

Coligan, J.E., Dunn, B.M., Speicher, D.W., and Wingfield, P.T. (eds.) 2002. Current Protocols in Protein Science. John Wiley & Sons, New York.

This book is advertised as "the chaperone to protein research." Because proteins are central to understanding food, the book is a must in food analytical chemistry.

Contributed by M. Angeles Navarrete del Toro and Fernando L. García-Carreño
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Electrophoresis Analysis

This unit describes polyacrylamide gel electrophoresis of protein samples. Proteins are important constituents of foods. They not only provide essential nutrients but also contribute to the functional properties needed to develop desired textural and sensory qualities of manufactured foods. During the manufacturing process, proteins can undergo many changes. Electrophoresis is the method of choice to gain insight into changes in protein composition, hydrolysis, or any modifications that may occur. Applications of protein electrophoresis include sample comparison; purity evaluation; determination of physical characteristics such as molecular weight, isoelectric point, and subunit composition; and the purification of small amounts of protein for further analysis. These widely used techniques are easily performed using inexpensive equipment.

In protein electrophoresis, a sample is applied to a polyacrylamide gel and its protein components are separated by application of an electric field across the gel. Separation is dependent on the charge and size of the proteins in the sample. Different approaches to this method have been developed to suit a variety of purposes.

In Basic Protocol 1, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate denatured and fully reduced proteins on the basis of their molecular weight. Alternate Protocol 1 describes the preparation of gradient gels, which allow for separation of a wider range of molecular weights than conventional homogeneous gels. In Alternate Protocol 2, native discontinuous PAGE is used for separation that depends both on the size and intrinsic charge of the proteins. Alternate Protocol 3, SDS-PAGE in a Tris-tricine buffer system, describes the electrophoretic separation of proteins and peptides in the range of 1 to 20 kDa. In Basic Protocol 2, native isoelectric focusing (IEF) is used to separate proteins on the basis of their isoelectric point (pI). Alternate Protocol 4 offers a denaturing version of this technique.

Several protocols in this unit describe methods for visualizing results following electrophoresis. Support Protocol 1 renders the protein bands visible with Coomassie brilliant blue R250 dye. Support Protocol 2 describes silver staining, a more complicated technique that provides exceptional sensitivity. Basic Protocol 3 describes the specific staining for proteinases of samples separated in polyacrylamide gels. Alternate Protocols 5 and 6 describe gel electrophoresis to detect proteinase inhibitors and the determination of proteinase class, respectively. Finally, Support Protocol 3 describes the estimation of protein molecular weights by SDS-PAGE.

CAUTION: Many of the following protocols utilize acrylamide monomer, a neurotoxin and suspected carcinogen, as well as other chemical substances that require special handling. When working with acrylamide, general handling procedures include using double latex gloves and weighing the material in a hood while wearing a disposable dust mask. See *APPENDIX 2B* for other handling guidelines.

CAUTION: Voltages and currents used for electrophoresis are potentially lethal. Use properly shielded, safety-certified equipment.

NOTE: Cleanliness and reagent quality are particularly important for electrophoresis. Always wear gloves when preparing electrophoresis reagents and handling electrophoresis equipment. Clean all assemblies that will contact the gels or sample with a detergent designed for laboratory glassware and rinse well with distilled water. Always use the highest-quality reagents and the purest distilled or deioinized water available.

Contributed by Tom Berkelman and Fernando García-Carreño

Current Protocols in Food Analytical Chemistry (2001) B3.1.1-B3.1.28

BASIC PROTOCOL 1

DENATURING DISCONTINUOUS PAGE (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weights. The technique is performed in polyacrylamide gels containing SDS. When proteins are treated with both SDS and a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol, separations exclusively by molecular weight are possible. The most commonly used buffer system for SDS-PAGE is the Tris-glycine system described below. An example of an SDS-PAGE gel is given in Figure B3.1.1.

Materials

30% (w/v) acrylamide solution (see recipe)
4× running gel buffer (see recipe)
10% (w/v) SDS (*APPENDIX 2A*)
10% (w/v) ammonium persulfate (make fresh)
Tetramethylethylenediamine (TEMED)
Water-saturated butanol (see recipe)
Running gel overlay(see recipe)
4× stacking gel buffer (see recipe)
Protein sample
2× SDS sample buffer (see recipe)
SDS-PAGE tank buffer, pH 8.3 (see recipe)
Protein molecular weight standards appropriate for gel percentage (optional)

Standard or mini-format vertical electrophoresis apparatus (e.g., Hoefer SE600, SE260, or miniVE; Amersham Pharmacia Biotech) with associated accessories, including casting stand, glass plates, 1-mm-thick spacers, and comb
Side-arm vacuum flasks with stoppers
Boiling water bath or 100°C heating block
Long, narrow gel-loading micropipettor tips
Power supply capable of delivering constant current at voltages ≥250 V

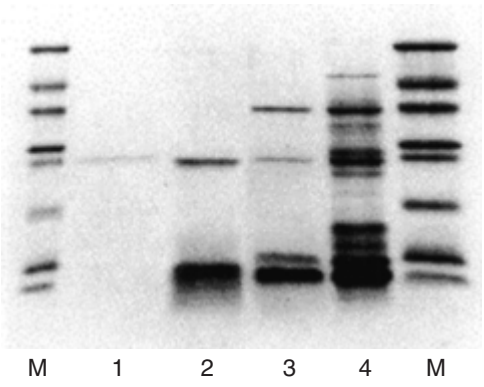

Figure B3.1.1 A 15% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Protein samples were assayed for the purification of a proteinase, cathepsin L, from fish muscle according to the method of Seymour et al. (1994). Lane 1, purified cathepsin L after butyl-Sepharose chromatography. Lane 2, cathepsin L complex with a cystatin-like proteinase inhibitor after butyl-Sepharose chromatography. Lane 3, sarcoplasmic fish muscle extract after heat treatment and ammonium sulfate precipitation. Lane 4, sarcoplasmic fish muscle extract. Lanes M, low-molecular-weight standards: aprotinin (M_r 6,500), α-lactalbumin (M_r 14,200), trypsin inhibitor (M_r 20,000), trypsinogen (M_r 24,000), carbonic anhydrase (M_r 29,000), gylceraldehyde-3-phosphate dehydrogenase (M_r 36,000), ovalbumin (M_r 45,000), and albumin (M_r 66,000) in order shown from bottom of gel. Lane 1 contains 4 μg protein; lanes 2 to 4 each contain ~7 μg protein.

Additional reagents and equipment for staining with Coomassie brilliant blue (see Support Protocol 1) or silver (see Support Protocol 2), and for estimating protein molecular weight (optional; see Support Protocol 3)

Prepare running gel

1. Assemble a gel sandwich (consisting of glass plates and 1-mm-thick spacers) into a casting stand according to manufacturer's instructions.

   The glass plates should be cleaned with a laboratory glassware cleaner such as RBS-35 (Pierce), rinsed well, and dried.

   This protocol can be used for either standard (14 × 16–cm) or mini-format (8 × 10–cm) gels. The recipes can be adjusted for 0.75 or 1.5-mm-thick gels. Alternatively, precast gels for SDS-PAGE, which are available from a number of manufacturers, can be used, and protocol should be started at step 16.

2. Determine the appropriate acrylamide percentage of running gel solution (see Table B3.1.1), and mix solution in a side-arm vacuum flask according to Table B3.1.2, leaving out the ammonium persulfate and TEMED.

3. Stopper flask and apply a water vacuum for several minutes while shaking or stirring to deaerate the solution.

4. Add ammonium persulfate and TEMED and gently swirl flask to mix, being careful not to generate bubbles.

Table B3.1.1 Recommended Acrylamide Concentrations for Protein Separation by SDS-PAGE

% Acrylamide in running gel	Separation size range (kDa)
Single percentage:	
5%	36-200
7.5%	24-200
10%	14-200
12.5%	14-100[a]
15%	14-60[a]
Gradient:	
5%-15%	14-200
5%-20%	10-200
10%-20%	10-150[a]

[a]Larger proteins move into the gel but fail to separate significantly.

Table B3.1.2 Running Gel Solutions for 1-mm-Thick Gels[a]

Component	Final percentage[b]				
	5%	7.5%	10%	12.5%	15%
30% Acrylamide solution	6.7 ml	10 ml	13.3 ml	16.7 ml	20 ml
4× Running gel buffer	10 ml	10 ml	10 ml	10 ml	10 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
H_2O	22.7 ml	19.4 ml	16.1 ml	12.7 ml	9.4 ml
10% Ammonium persulfate[c]	200 µl	200 µl	200 µl	200 µl	200 µl
TEMED[c]	15 µl	15 µl	15 µl	15 µl	15 µl

[a]Final volume is 40 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add after deaeration (step 3).

5. Pipet solution down the spacer and into gel sandwich to a level 3 to 4 cm from the top.
6. Overlay gel with a thin layer (100 to 500 µl) of water-saturated butanol and allow to polymerize.

   A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10 to 20 min. The gel should be fully polymerized after 1 to 2 hr.

7. Tilt casting stand to pour off butanol and rinse gel surface once with running gel overlay.
8. Overlay gel with running gel overlay and allow gel to sit while preparing stacking gel.

Prepare stacking gel

9. Prepare stacking gel solution according to Table B3.1.3 in a side-arm vacuum flask, leaving out the ammonium persulfate and TEMED.
10. Deaerate as in step 3.
11. Add ammonium persulfate and TEMED. Gently swirl flask to mix, being careful not to generate bubbles.
12. Pour off running gel overlay.
13. Add 1 to 2 ml stacking gel solution to gel sandwich to rinse the gel surface. Rock casting stand and pour off liquid.
14. Fill gel sandwich with stacking gel solution and insert a comb into the sandwich, taking care not to trap any bubbles below the comb teeth.
15. Allow gel to polymerize ≥60 min.

    A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10 to 20 min. The gel should be fully polymerized after 1 to 2 hr. In general, stacking gels should be cast just before use. However, the complete gel can be stored overnight at 4°C, with little effect on resolution, if covered with the comb in place.

Prepare sample

16. Combine equal volumes protein sample and 2× SDS sample buffer and incubate 90 sec in a boiling water bath or 100°C heating block.

    If the gels will be stained with Coomassie brilliant blue (see Support Protocol 1), a starting sample protein concentration of 10 to 20 mg/ml should be used. This will be diluted by the 2× SDS sample buffer to give 5 to 10 µg/µl. For complex mixtures, 50 µg protein (5 to 10 µl treated sample) per lane is recommended. For highly purified proteins, 0.5 to 5 µg per lane is usually adequate. Silver staining (see Support Protocol 2) requires 10- to 100-fold less protein per lane.

Table B3.1.3 Stacking Gel Solution (4% Acrylamide) for 1-mm-Thick Gels[a]

Component	Volume
30% Acrylamide solution	2 ml
4× Stacking gel buffer	3.75 ml
10% SDS	150 µl
H_2O	9 ml
10% Ammonium persulfate[b]	75 µl
TEMED[b]	7.5 µl

[a]Final volume is 15 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) gels.
[b]Added after deaeration (step 10).

17. Place sample on ice until ready for use.

    The treated sample can be stored at –20°C for 6 months for future runs.

Load gel

18. Slowly remove comb from the gel, angling the comb up to avoid disturbing the well dividers

19. Rinse each well with SDS-PAGE tank buffer, invert the casting stand to drain the wells, and return the stand to an upright position.

20. Fill each well with SDS-PAGE tank buffer.

21. Using a micropipettor fitted with a long, narrow gel-loading tip, gently and slowly load 5 to 10 µl sample beneath the buffer in each well. Load every well with the same volume of sample. If a well is not needed, load it with 1× SDS sample buffer containing standard protein or no sample.

    This procedure ensures that each well behaves the same during separation. If a well is left empty, the adjacent samples will tend to spread during electrophoresis.

    When adding the sample, a sharp interface should be maintained between the sample and the SDS-PAGE tank buffer. Adding the sample too fast or erratically will lead to swirling and a diffuse loading zone. This will cause a loss of band sharpness.

    Depending on the design of the gel apparatus, it may be preferable to load the gel following installation of the gel into the apparatus and addition of buffer to the upper buffer chamber (steps 23 to 25).

22. If protein molecular weight standards are used, follow manufacturer's instructions for their preparation and load one or two wells with 5 to 10 µl standards.

    This volume should contain 0.2 to 1 µg of each standard component if the gel is to be stained with Coomassie brilliant blue, and ~10 to 50 ng of each component if the gel is to be silver stained.

Run gel

23. Fill lower buffer chamber of the electrophoresis apparatus with SDS-PAGE tank buffer.

24. Remove gel sandwich from casting stand and install it in the electrophoresis apparatus according to manufacturer's instructions. Make sure bottom of gel cassette is free of bubbles.

25. Carefully fill upper buffer chamber with SDS-PAGE tank buffer. Do not pour buffer into the sample wells because it will wash the sample out.

26. Put safety lid on the gel apparatus and connect it to a power supply.

    The cathode (black lead) is connected to the upper buffer chamber.

27. Set power supply to constant current and turn it on. Adjust current to 20 mA per gel.

    When running 0.75- or 1.5-mm-thick gels, the current should be adjusted accordingly; 0.75-mm-thick gels should be run at 15 mA per gel, and 1.5-mm-thick gels at 30 mA per gel.

28. Keep a record of the voltage and current readings to compare with future runs and to detect current leaks or incorrectly made buffers.

    The voltage should start at ~70 to 80 V, but will increase during the run.

Under these conditions, a standard (14 × 16–cm) gel will take ~5 hr to run and a mini-format (8 × 10–cm) gel will take ~1.5 hr. If it is more convenient to run the gel for a longer period, e.g., 10 hr for a standard gel, the current should be cut in half (to 10 mA/gel). For a 15-hr run (i.e., overnight), the current should be cut to 7 mA/gel.

If an electrophoresis apparatus with active cooling capability is used, considerably higher currents (up to 50 mA per gel) can be used, with correspondingly shorter run times.

29. When the dye front reaches the bottom of the gel, turn power supply off and disconnect power cables.

30. Visualize protein bands by Coomassie brilliant blue (see Support Protocol 1) or silver staining (see Support Protocol 2). To estimate protein molecular weight, see Support Protocol 3.

ALTERNATE PROTOCOL 1

LINEAR GRADIENT PAGE

Gradient gels, although more difficult to cast than single-concentration gels, fractionate a wider size range of proteins on a single gel. Furthermore, calculating molecular weights (see Support Protocol 3) is simplified because, unlike single-concentration gels, the relationship between the logarithm of the molecular weight of a protein and its mobility is linear over most of the fractionation range of a gradient gel. A gradient maker mixes a high- and a low-percentage acrylamide solution during the casting process, which results in a gel that spans a range of acrylamide percentages. The protocol below describes casting one gradient gel at a time. A multiple gel caster can cast multiple gradient gels simultaneously. Consult the manufacturer's instructions for casting several gradient gels at once.

Additional Materials *(also see Basic Protocol 1)*

Sucrose
Pump tubing
Side-outlet gradient maker for linear gradients (e.g., Hoefer SG50 for standard gels and Hoefer SG15 for mini-format gels; Amersham Pharmacia Biotech)
Peristaltic pump capable of delivering 1 to 6 ml/min

Set up gradient maker

1. Assemble a gel sandwich as described above (see Basic Protocol 1, step 1).

2. Connect a piece of pump tubing to the outlet tubing connector of a side-outlet gradient maker.

Table B3.1.4 Light Gradient Running Gel Solutions[a]

Component	Final percentage[b]				
	5%	7.5%	10%	12.5%	15%
30% Acrylamide solution	3.3 ml	5.0 ml	6.7 ml	8.3 ml	10.0 ml
4× Running gel buffer	5 ml	5 ml	5 ml	5 ml	5 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
H_2O	11.3 ml	9.6 ml	7.9 ml	6.3 ml	4.7 ml
10% Ammonium persulfate	180 µl	160 µl	130 µl	110 µl	80 µl
TEMED[c]	6.6 µl	6.6 µl	6.6 µl	6.6 µl	6.6 µl

[a]Final volume is 20 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add just before pouring the gel (step 7).

3. Force a 1 to 200 µl pipet tip onto the other end of the tubing and, using a ring stand and clamp, position the tip 0.5 to 1 mm inside the top center of the gel sandwich. Use the clamp to hold the pipet tip firmly in place.

4. Attach tubing to a peristaltic pump.

Prepare gel solutions

5. Determine appropriate low and high percentages of the gradient gel (see Table B3.1.1). In separate flasks, mix all ingredients listed in Tables B3.1.4 and B3.1.5 for the respective light and heavy gradient running gel solutions, including ammonium persulfate. Do not add TEMED.

   A 5% to 20% or 10% to 20% gradient gel is recommended.

6. Gently swirl flasks to mix and place heavy gradient solution on ice.

   Deaeration is not needed in this protocol.

   The heavy gradient solution should be placed on ice to prevent polymerization. Polymerization can occur in high-concentration acrylamide solutions without addition of TEMED once ammonium persulfate has been added.

7. Add TEMED and gently swirl flasks to mix.

   Alternatively, gel solution can be added to the gradient maker before TEMED is added. TEMED (0.33 µl/ml gel solution) can be added just before opening the outlets and mixed by drawing the solution in and out of a disposable plastic pipet. If this technique is used, a large volume of heavy and light solutions can be prepared in advance and dispensed into the gradient maker for each individual gel. This is useful when casting several gels individually without using a multiple gel caster.

Pour gel

8. Pour heavy gradient solution into the chamber closest to the outlet (mixing chamber) of the gradient maker and add a small stir-bar. Use 9.3 ml per chamber for 1-mm-thick standard (14 × 16–cm) gels. Use 2.3 ml per chamber for 1-mm-thick mini-format (8 × 10–cm) gels.

   Volumes can be adjusted accordingly if pouring 0.75 or 1.5-mm-thick gels.

9. Open stopcock between the two chambers and allow a small amount of heavy gradient solution to flow through the channel to, but not into, the bottom of the reservoir chamber. Close stopcock.

10. Pour light gradient solution into the reservoir chamber.

Table B3.1.5 Heavy Gradient Running Gel Solutions[a]

Component	Final percentage[b]				
	10%	12.5%	15%	17.5%	20%
30% Acrylamide solution	6.7 ml	8.3 ml	10.0 ml	11.7 ml	13.3 ml
4× Running gel buffer	5 ml	5 ml	5 ml	5 ml	5 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Sucrose	3 g	3 g	3 g	3 g	3 g
H_2O	6.46 ml	4.66 ml	3.06 ml	1.40 ml	—
10% Ammonium persulfate	100 µl	90 µl	40 µl	40 µl	40 µl
TEMED[c]	6.6 µl	6.6 µl	6.6 µl	6.6 µl	6.6 µl

[a]Final volume is 20 ml, sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]See Table B3.1.1 for guidelines on selecting appropriate percentage.
[c]Add just before pouring the gel (step 7).

11. Place the gradient maker on a magnetic stirrer and begin stirring.
12. Turn on the pump and open the outlet stopcock.

    The pump rate should be set so that casting takes from 5 to 10 min.

13. Open stopcock between the two chambers. Continue to pump until all liquid is in the gel sandwich.
14. Overlay gel with 100 µl water-saturated butanol and allow gel to polymerize.
15. Continue with casting the stacking gel and running the sample (see Basic Protocol 1, steps 7 to 30).

ALTERNATE PROTOCOL 2

NATIVE DISCONTINUOUS PAGE

Under native conditions, polypeptides retain their higher-order structure and often retain enzymatic activity and interactions with other polypeptides. The migration of proteins under native conditions depends on many factors including size, shape, and native charge. One straightforward approach to native electrophoresis is to leave out the SDS and reducing agent (DTT or 2-mercaptoethanol) from the SDS-PAGE protocol (see Basic Protocol 1). Prepare all solutions without SDS, including tank buffer. Leave out both SDS and reductant from 2× sample buffer, and do not heat treat samples. Figure B3.1.2 shows an example of a native, discontinuous polyacrylamide gel.

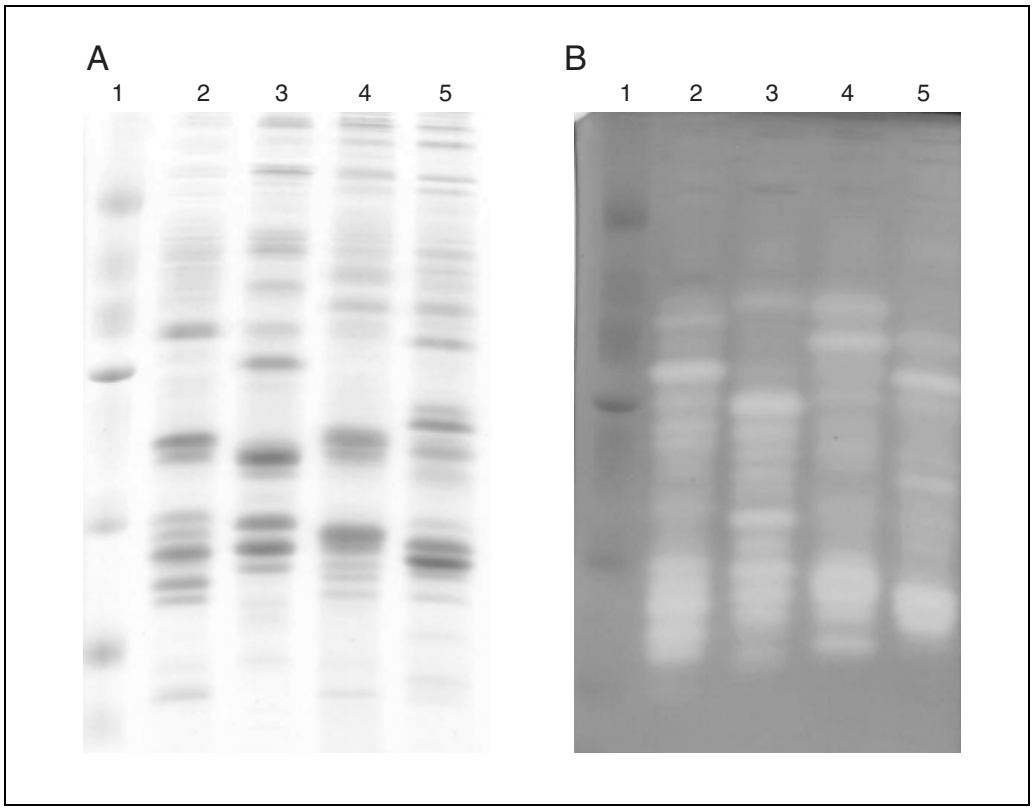

Figure B3.1.2 Native discontinuous polyacrylamide gels activity stained for proteinases. (**A**) Gel stained with Coomassie brilliant blue for total protein. (**B**) Gel assayed for proteinase activity using casein as a substrate. Samples are enzyme extracts of hepatopancreas from four shrimp species. Lane 1, molecular weight markers; Lane 2, *P. californiensis*; Lane 3 *P. vannamei*; Lane 4, *P. paulensis*, Lane 5, *P. schmitti*.

SDS-PAGE IN A TRIS-TRICINE BUFFER SYSTEM

ALTERNATE PROTOCOL 3

The Tris-glycine discontinuous buffer system of Laemmli cannot be used for the separation of proteins with molecular weights <10 to 15 kDa. For the analysis of smaller proteins, an alternative Tris-tricine buffer system is used along with an acrylamide solution that has a high percentage of cross-linker. This technique should be used when separating peptides in the size range of 1 to 20 kDa.

Additional Materials *(also see Basic Protocol 1)*

Ethylene glycol
40% (w/v) acrylamide solution (see recipe)
4× Tris-tricine gel buffer (see recipe)
Upper (cathodic) Tris-tricine tank buffer (see recipe)
Lower (anodic) Tris-tricine tank buffer (see recipe)

1. Prepare gel solutions and pour a gel for the Tris-tricine system as described above (see Basic Protocol 1, steps 1 to 15), but use the 16% running gel and 5% stacking gel solutions given in Table B3.1.6.

2. Prepare sample and load onto gel (see Basic Protocol 1, steps 16 to 22), but use upper (cathodic) Tris-tricine tank buffer to load the sample in steps 19 and 20.

   A different sample buffer may be recommended in some literature, but the author sees no difference when the standard sample buffer is used for this technique.

3. Run the gel (see Basic Protocol 1, steps 23 to 30), but fill the lower and upper buffer chambers of the electrophoresis apparatus with lower (anodic) and upper (cathodic) Tris-tricine tank buffers, respectively, and adjust current to 70 mA per gel.

   Under these conditions, a standard (14 × 16-cm) gel will take 5 to 6 hr to run and a mini-format (8 × 10-cm) gel will take 2 to 2.5 hr.

Table B3.1.6 Gel Solutions for Tris-Tricine System[a]

Component	16% Running gel	5% Stacking gel
Ethylene glycol	14 ml	—
40% Acrylamide solution	16 ml	—
30% Acrylamide solution	—	2.5 ml
4× Tris-tricine gel buffer	10 ml	3.75 ml
H_2O	—	8.7 ml
10% Ammonium persulfate[b]	160 µl	60 µl
TEMED[b]	20 µl	7.5 µl

[a]Sufficient for two standard (14 × 16–cm) or four mini-format (8 × 10–cm) 1-mm-thick gels.
[b]Add after deaeration.

NATIVE ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) separates proteins according to their isoelectric point (pI). The technique is performed in thin polyacrylamide gels incorporating carrier ampholytes, which are small amphoteric compounds that form a pH gradient when voltage is applied across the gel. Proteins introduced into this pH gradient will migrate until they reach their isoelectric point. Each protein species in the sample can be visualized as a sharply focused band. IEF is best performed in a flatbed electrophoresis apparatus. This type of apparatus allows very effective cooling, which is necessary due to the high voltages employed for IEF. The following protocol is used to separate proteins under native conditions in a range from pH 3.5 to 9.5 in a commercially available precast IEF gel. An example of an IEF gel is given in Figure B3.1.3.

Materials

Protein sample
Kerosene or mineral oil
IEF anode solution: 1 M H_3PO_4 (*APPENDIX 2A*)
IEF cathode solution: 1 M NaOH (*APPENDIX 2A*)
Protein pI standards (prepared according to manufacturer's instructions)

Flatbed electrophoresis apparatus (e.g., Multiphor II; Amersham Pharmacia Biotech)
Thermostatic circulator
Precast IEF gel on plastic backing (e.g., Ampholine PAGplate, pH 3.5 to 9.5; Amersham Pharmacia Biotech)
IEF electrode strips (e.g., Amersham Pharmacia Biotech)
High-voltage power supply capable of delivering 1500 V while limiting both current and power
IEF sample applicator strip (e.g., Amersham Pharmacia Biotech)

Additional reagents and equipment for staining with Coomassie brilliant blue (see Support Protocol 1) or silver stain (see Support Protocol 2)

Prepare sample

1. Adjust sample concentration to 0.5 to 10 mg/ml for Coomassie brilliant blue staining or 0.05 to 1 mg/ml for silver staining.

   The composition at the sample buffer is not critical, but the ionic strength should be as low as possible. Buffers and salts should not be present at a concentration >50 mM.

   Sample solubility can occasionally be improved with the use of a nondenaturing, neutral detergent (e.g., 0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside) or polyalcohol (e.g., 20% [w/v] glycerol or sorbitol) in both the sample and the gel.

2. If sample contains insoluble materials, centrifuge 5 min at maximum speed in a microcentrifuge and transfer supernatant to a clean tube.

Prepare apparatus and gel

3. Connect a flatbed electrophoresis apparatus to a thermostatic circulator and set the temperature to 10°C. Turn on the thermostatic circulator 10 min before starting the analysis.

4. Pipet ~1 ml kerosene or mineral oil onto the cooling plate of the electrophoresis apparatus.

5. Remove a precast IEF gel on plastic backing from its package and position it in the center of the cooling plate, allowing the kerosene or mineral oil to spread evenly underneath the gel. Make sure no bubbles are trapped beneath the gel.

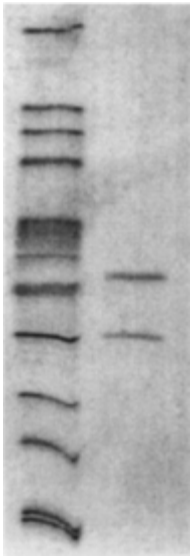

Figure B3.1.3 An isoelectric focusing (IEF) gel, pH 3 to 10. Lane 1, ~4 μg purified egg white cystatin. Lane M, broad-range pI standards: trypsinogen (pI 9.3), lentil lectin-basic band (pI 8.65), lentil lectin-middle band (pI 8.45), lentil lectin-acidic band (pI 8.15), myoglobin-basic band (pI 7.35; visible as a broad band), myoglobin-acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase (pI 5.85), α-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.50) in order shown from top of gel. The pI values of the two purified egg white cystatin isomers were determined to be 6.6 (upper band) and 5.8 (lower band). Adapted from Akpinar (1998) with permission from author.

The gel can be used in one piece or, depending on the number of samples, cut into portions with scissors. If this is done, current and power limits should be reduced accordingly (e.g., if only half a gel is to be run, current and power limits should be set at 25 mA and 15 W, respectively, rather than 50 mA and 30 W). Unused portions of gel can be wrapped in plastic or placed in sealed plastic bags and stored at 4°C until use.

6. Soak one IEF electrode strip in ~3 ml IEF anode solution. Remove excess solution with lint-free tissues.
7. Repeat step 6 with a second IEF electrode strip using IEF cathode solution.
8. Apply electrode strips over the long edges of the gel, placing the electrode strip soaked with the anodic solution towards the anodic (+) side of the cooling plate.
9. Use sharp scissors to cut off the ends of the electrode strips that protrude beyond the ends of the gel.

Prerun gel (optional)

10. Place the electrode holder with the IEF electrodes on the electrophoresis apparatus and align electrodes over the center of the electrode strips. Connect the two electrodes to the base unit and place the lid on the apparatus.
11. Connect the leads to a high-voltage power supply. Set power supply to 1500 V, limiting the current at 50 mA and the power at 30 W. Run gel for 15 min.

The voltage should start out between 300 and 600 V and rise steadily towards 1500 V during the prerun step.

Prerunning the gel is optional, but can result in a higher-quality separation.

Apply sample and run gel

12. Turn off power supply and disconnect leads. Take lid off apparatus and remove electrode holder.

13. Lay an IEF sample applicator strip across the gel towards either the anodic or cathodic edge. Check that contact between gel and applicator strip is uniform.

    The optimal position for sample application varies with sample type and must be determined empirically. This is best done using sample application pieces (e.g., Amersham Pharmacia Biotech), which are 5 × 10–mm pads of absorbent material that can be placed in multiple positions on a single gel.

14. Pipet 5 to 20 µl sample and an appropriate volume of protein pI standards (if desired) into the wells in the applicator strip.

    The protein standards should contain 0.2 to 1 µg of each standard component if the gel is to be stained with Coomassie brilliant blue and ~20 to 100 ng of each component if the gel is to be silver stained.

15. Repeat steps 10 and 11, running the gel for 1.5 hr.

    The running time depends on the pH range of the gel. For gels covering other pH ranges, the manufacturer's instructions should be consulted.

16. Stain gel with Coomassie brilliant blue or silver stain to visualize the proteins (see Support Protocols 1 and 2, respectively).

    A plot of pI versus distance migrated from the cathode end of the gel can be constructed for the pI standard. This can be used to estimate the pI of the proteins in the sample.

ALTERNATE PROTOCOL 4

DENATURING ISOELECTRIC FOCUSING

In some cases, it is advantageous to perform IEF in the presence of 8 M urea. This denaturant renders some proteins more soluble under IEF conditions, allowing the analysis of samples that cannot be separated under native conditions. The following protocol separates proteins under denaturing conditions in a range from pH 3 to 10 using a commercially available dried polyacrylamide gel.

Additional Materials *(also see Basic Protocol 2)*

Dried polyacrylamide gel on plastic backing (e.g., CleanGel IEF; Amersham Pharmacia Biotech)
Denaturing IEF rehydration solution, pH 3 to 10 (see recipe)
Rehydration tray (e.g., GelPool; Amersham Pharmacia Biotech)
Rotary laboratory shaker
Filter paper
High-voltage power supply (capable of delivering 2500 V while limiting both current and power)

Rehydrate gel

1. Open gel package and remove a dried polyacrylamide gel on plastic backing.

   The dry gel can be used in one piece or, depending on the number of samples, cut into portions with scissors. Unused portions of gel can be wrapped in plastic or placed in sealed plastic bags and stored at –20°C until use.

2. Select the appropriate chamber of a rehydration tray. Clean tray with water and dry with lint-free tissues.

3. Pipet denaturing IEF rehydration solution into the chamber. For a full-size gel, use 10.4 ml. For portions of the gel, reduce volume accordingly (e.g., use 5.2 ml for half a gel).
4. Set the edge of the dried gel, with the gel surface downwards, into the rehydration solution and slowly lower it. Lift gel at the edges with forceps and slowly lower it down again to ensure an even distribution of liquid and to remove air bubbles.
5. Place rehydration tray on a rotary laboratory shaker and shake gently. Allow gel to rehydrate 4 hr to overnight.

   The gel must be used immediately following rehydration.

Prepare sample and run gel

6. Dissolve or dilute protein sample into denaturing IEF rehydration solution to a concentration of 1 to 3 mg/ml for Coomassie brilliant blue staining or 10 to 300 µg/ml for silver staining.

   The composition of the sample solution should be as similar as possible to the composition of the rehydration solution. Buffers and salts should not be present at a concentration >50 mM.

7. Continue to prepare sample and gel as described (see Basic Protocol 2, steps 2 to 5), except set the thermostatic circulator to 15°C, and remove gel from the rehydration tray and carefully dry gel surface with the edge of a sheet of filter paper before positioning it on the cooling plate.

   The gel surface should be absolutely dry.

8. Place an electrode holder with IEF electrodes on electrophoresis apparatus and align electrodes so that they rest on the outer edges of the gel. Connect the two electrodes to the base unit and place lid on apparatus.

   IEF electrode strips or wicks are not necessary.

9. Connect leads to a high-voltage power supply. Prerun gel for 20 min following the conditions given in Table B3.1.7.
10. Apply sample and run gel as described (see Basic Protocol 2, steps 12 to 16), except align electrodes in step 15 so that they rest on the outer edges of the gel and follow the recommended settings in Table B3.1.7.

Table B3.1.7 Running Conditions for Denaturing IEF

Step	Voltage (V)	Current (mA)[a]	Power (W)[a]	Time (min)
Prerun	700	12	8	20
Sample entrance	500	8	8	20
Isoelectric focusing	2000	14	14	90
Band sharpening	2500	14	18	10

[a]If only a portion of the gel is run, reduce current and power limits accordingly.

SUPPORT PROTOCOL 1

COOMASSIE BRILLIANT BLUE STAINING OF POLYACRYLAMIDE GELS

Coomassie brilliant blue staining is based on nonspecific binding of Coomassie brilliant blue dye to proteins. Separated proteins are simultaneously fixed and stained in the gel, and then destained to remove background staining prior to drying and documenting. The proteins are detected as blue bands on a clear background. The standard protocol can be used to stain conventional SDS-PAGE or native gels as well as IEF and Tris-tricine gels. When staining conventional SDS-PAGE or native gels, a fixing step is not required. A gel stained with Coomassie brilliant blue is shown in Figure B3.1.1.

Materials

Polyacrylamide gel (SDS-PAGE, IEF, native PAGE, or Tris-tricine SDS-PAGE) containing separated proteins (see Basic Protocols 1 and 2; see Alternate Protocols 1 to 4)
20% (w/v) trichloroacetic acid (TCA; for IEF gels only; store ≤1 month at room temperature)
Destaining solution I: 40% (v/v) methanol/7% (v/v) acetic acid (store ≤1 month at room temperature)
Glutaraldehyde fixing solution (see recipe; for Tris-tricine gels only)
0.025% (w/v) Coomassie brilliant blue staining solution (see recipe)
Destaining solution II: 5% (v/v) methanol/7% (v/v) acetic acid (store indefinitely at room temperature)
Glycerol

Covered tray
Laboratory shaker or rocker
Filter paper (e.g., Whatman 3MM) or porous cellophane sheets (e.g., Amersham Pharmacia Biotech).
Vacuum gel dryer (e.g., Hoefer GD2000; Amersham Pharmacia Biotech) attached to vacuum pump or air gel dryer with dryer with drying frames (e.g., Hoefer Easy Breeze; Amersham Pharmacia Biotech)

1a. *For IEF gels:* Place a polyacrylamide gel in a covered tray with enough 20% TCA to fully submerge the gel. Shake slowly 30 to 60 min on a laboratory shaker or rocker. Replace 20% TCA with destaining solution I and shake slowly for 3 min. Remove destaining solution I.

Do not leave a gel in 20% TCA for >60 min.

1b. *For Tris-tricine gels:* Place a polyacrylamide gel in a covered tray with enough glutaraldehyde fixing solution to fully submerge the gel. Shake slowly 30 to 60 min on a laboratory shaker or rocker. Remove fixing solution.

1c. *For SDS-PAGE and native PAGE gels:* Place a polyacrylamide gel in a covered tray.

2. Add just enough Coomassie brilliant blue staining solution so that the gel floats freely in the tray. Shake slowly ~4 hr to overnight on a laboratory shaker or rocker.

CAUTION: *Exposure to methanol and acetic acid vapors is minimized when using covered plastic trays. When covers are not used, these procedures should be done in a fume hood.*

For accelerated staining and destaining, the solutions can be heated to 45°C, which will reduce the time by 50%.

3. Replace staining solution with destaining solution I. Shake slowly 30 min.

This removes the bulk of the excess stain.

4. Remove destaining solution I and replace with destaining solution II. Change destaining solution II periodically until gel background is clear, typically ≤8 hr.

Alternatively, the waste volume can be minimized by the addition of paper tissue to one corner of the staining tray. Coomassie brilliant blue is removed from the gel without changing the destaining solution, minimizing the waste volume generated. The tissues are replaced when they are saturated with Coomassie brilliant blue. Caution should be used, however, because excessive destaining will lead to loss of band intensity.

5. Store gel in destaining solution II ≤1 week in a covered tray. To minimize cracking, add 4% (v/v) glycerol to the last destaining solution before drying the gel.

   For longer term storage, gels may be stored wet at 4°C. The wet gel should be wrapped in a piece of plastic wrap. This permits handling the gel without risk of breakage. The wrapped gel can be stored in a sealable bag for ≤1 year at 4°C. To preserve gels indefinitely, they may be either vacuum dried onto filter paper or air dried between sheets of cellophane.

6a. *For vacuum drying gels:* Place gel on a sheet of filter paper of the same size. Place gel and paper on a larger sheet of filter paper covering the metal screen of the platen of a vacuum gel dryer attached to a vacuum pump. Cover top of gel with plastic wrap, lower silicon cover flap, apply vacuum to seal flap, and turn on heater. Dry gel, typically <2 hr.

   During vacuum drying, the gel will feel cold relative to the surrounding platen if it is not completely dry. When the gel temperature is the same as the platen, the gel is dry and the vacuum and dryer can be turned off. Gel cracking can be caused by releasing the vacuum before the gel is dry.

6b. *For air drying gels:* Place gel between two sheets of porous cellophane and lock into the drying frame of an air dryer. Insert frame into air dryer and turn on fan and heater. Dry gel, typically <2 hr.

   Moisture evaporates through the cellophane leaving a flat, easy-to-store gel with a clear background.

   As an alternative to drying, gels may be photographed with a Polaroid or digital camera. Illumination should be provided by placing the gel on a light box. Gels may also be scanned with a transparency scanner.

SILVER STAINING OF POLYACRYLAMIDE GELS

SUPPORT PROTOCOL 2

Silver staining is approximately 50-fold more sensitive than Coomassie brilliant blue staining (see Support Protocol 1). It is a complex, multistep process, and many variables can influence the results. High-purity reagents and precise timing are essential for reproducible, high-quality results. Impurities in the gel and/or the water used for preparing the staining reagents can give poor staining results. The detection limit of this technique is from 1 to 5 ng protein per band. This protocol gives the best results when applied to standard SDS-PAGE and native gels. Silver staining IEF gels is less sensitive and high levels of background staining can be expected unless extra steps are taken to remove interfering carrier ampholytes. Tris-tricine gels can be silver stained according to this procedure, but a glutaraldehyde fixing solution (see Support Protocol 1), which prevents the loss of small peptides, cannot be used. Therefore, small peptides may diffuse out of the gel during staining and be lost.

Materials

Polyacrylamide gel (SDS-PAGE, IEF, native PAGE, or Tris-tricine SDS-PAGE) containing separated proteins (see Basic Protocols 1 and 2; see Alternate Protocols 1 to 4)
Silver stain fixing solution: 40% (v/v) ethanol/10% (v/v) acetic acid (make fresh)
Sensitizing solution (see recipe)
Silver solution (see recipe)

Table B3.1.8 Silver Staining Protocol

Solution	Time (≤1-mm-thick gel)	Time (1.5-mm-thick gel)
Silver stain fixing solution[a]	30 min	30 min
Sensitizing solution	30 min	30 min
H_2O	3 × 5 min	3 × 10 min
Silver solution	20 min	30 min
H_2O	2 × 1 min	2 × 1 min
Developing solution[b]	3-5 min	5-10 min
Stop solution	10 min	10 min
H_2O	3 × 5 min	3 × 5 min
Preserving solution[c]	30 min	30 min

[a]Leave gel in this solution until a convenient time for completing procedure (≤1 week).
[b]Monitor development and change solution when protein bands are visible and background is just starting to darken.
[c]Store gel in this solution ≤1 week. The glycerol will prevent gel from cracking when dried.

Developing solution (see recipe)
Stop solution: 1.5% (w/v) Na_2EDTA (store ≤6 months at room temperature)
Preserving solution: 30% (v/v) ethanol/4% (v/v) glycerol (store ≤6 months at room temperature)

Covered tray
Laboratory shaker or rocker

Additional equipment for drying gel (see Support Protocol 1)

NOTE: Clean all equipment used for running and staining the gel with detergent and rinse thoroughly. Wear clean gloves when handling the electrophoresis apparatus, the gel, or the staining tray.

Place a polyacrylamide gel in a covered tray and process according to Table B3.1.8. Use 250 ml of each solution per standard (14 × 16–cm) gel and 100 ml per mini-format (8 × 10–cm) gel. Shake slowly on a laboratory shaker or rocker during each step. When staining an IEF gel, begin with a 30-min fixation step in 20% TCA (see Support Protocol 1) and then proceed with steps described in Table B3.1.8, adding three additional washes with water between the sensitizing step and silver step if needed. The timing of some steps differs according to the thickness of the gel as indicated in Table B3.1.8. If desired, dry gel as described (see Support Protocol 1, step 6).

BASIC PROTOCOL 3

ACTIVITY STAINING FOR PROTEINASE

Proteinases in complex protein samples are separated and detected by their activity using a technique called substrate-SDS-PAGE. After electrophoresis, the gel is incubated in a casein substrate solution and then stained with Coomassie brilliant blue. The presence of active proteinase is indicated by clear zones on a blue background. Information about the number of active components and their molecular weights is obtained by this technique. This method is useful when studying the presence, diversity, amount, and class of proteinases in food ingredients that affect properties such as texture. Other measurements of proteinase activity are addressed in *UNIT C2.1*. Figure B3.1.2 shows a discontinuous polyacrylamide gel activity stained for proteinase.

Materials

Proteinase-containing protein sample
2 × nonreducing sample buffer (see recipe)
2% casein (added just before use) in 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.1% (w/v) Coomassie brilliant blue staining solution (see recipe)
Destaining solution: 40% (v/v) ethanol/10% (v/v) acetic acid (optional; store ≤1 month at room temperature)

Additional reagents and equipment for SDS-PAGE (see Basic Protocol 1) and Coomassie brilliant blue staining and drying gel (see Support Protocol 1).

1. Dilute proteinase-containing protein sample 1:1 (v/v) in 2 × nonreducing sample buffer.

   The amount of sample used will depend on its proteolytic activity. The author recommends measuring this activity using azocasein as a substrate (García-Carreño, 1992). A sample with 10 mU of activity should be loaded.

2. Prepare and run duplicate SDS-polyacrylamide gels as described (see Basic Protocol 1, steps 1 to 29), using the diluted proteinase-containing protein sample and appropriate molecular weight standards. Do not boil the diluted sample.

   A mini-format (8 × 10–cm) gel is recommended.

   The duplicate gel will be used to calibrate molecular weights and visualize total protein.

3. Disassemble gel cassettes, place gels in separate covered trays, and immerse one gel in 50 ml of 2% casein in 50 mM Tris·Cl for 30 min at 4°C. Stain second gel with Coomassie brilliant blue as described (see Support Protocol 1, steps 1 to 5).

   This incubation allows the substrate to diffuse into the gel at reduced enzyme activity.

   The Coomassie staining solution is acidic enough to stop proteinase activity.

4. Move the casein gel to 25°C (room temperature) and incubate ≤90 min.

   Substrate is digested at sites where proteinases are located.

5. Wash casein gel briefly with water.

6. Place casein gel in covered tray containing enough 0.1% Coomassie brilliant blue staining solution to cover gel. Shake slowly 2 hr on a laboratory shaker or rocker.

   Clear zones on a blue background, indicative of casein hydrolysis by proteinases, should be observable at this stage in the casein gel. Molecular weight standards, proteins other than proteinases, and inhibitor proteins are recognized by their blue color, which is of a higher intensity than the background caused by staining of the undigested casein.

7. *Optional:* Wash 2 hr in destaining solution.

   This step will improve the contrast of the clear zones.

8. Dry gel or record results as described (see Support Protocol 1, step 6).

DETECTION OF PROTEINASE INHIBITORS

ALTERNATE PROTOCOL 5

Proteinaceous proteinase inhibitors (present for example in legumes, eggs, and plasma) are separated and detected in complex mixtures by using a variation of the technique described in Basic Protocol 3. This technique is useful when studying the presence, diversity, amount, and specificity of proteinase inhibitors in food ingredients that affect protein digestion. An example of detection of proteinase inhibitors is given in Figure B3.1.4.

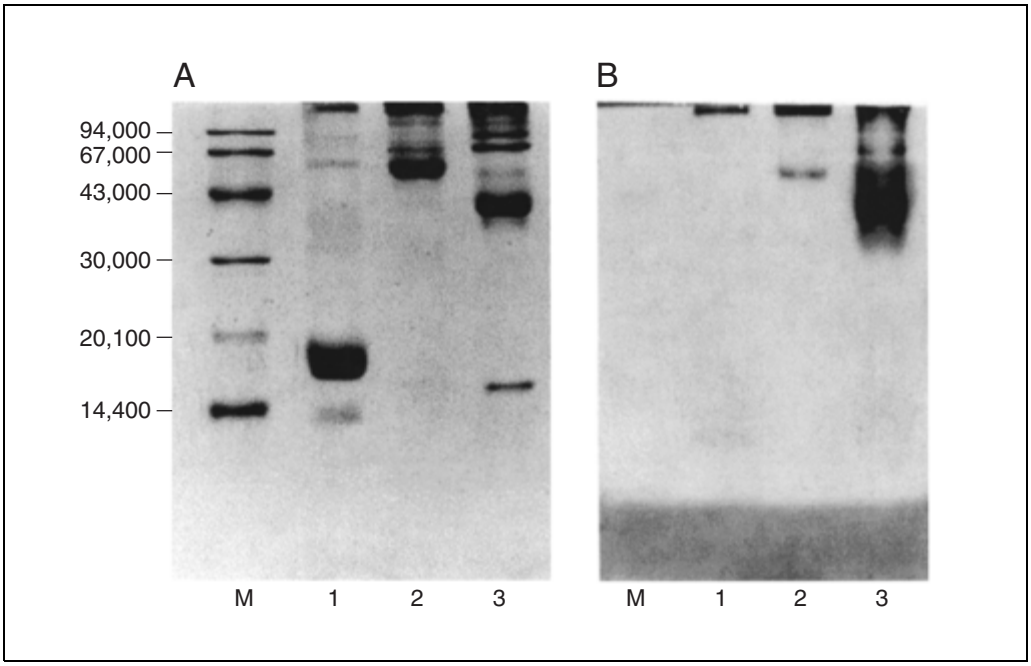

Figure B3.1.4 A 15% SDS-polyacrylamide gel assayed for proteinase inhibitors. (**A**) Gel stained with Coomassie brilliant blue for total protein. (**B**) Gel assayed for serine-proteinase inhibitory activity against trypsin. Food-grade proteinase inhibitors used in surimi manufacture were assayed. Lane 1, whey protein concentrate. Lane 2, bovine plasma proteins. Lane 3, egg white. Each lane contains 15 μg protein. Lane M, molecular weight standards. The dark bands in (B) indicate proteins with proteinase inhibitory activity. Numerous proteins in egg white (lane 3) are shown to have inhibitory activity against trypsin. Adapted from Weerasinghe (1995).

Additional Materials *(also see Basic Protocol 3)*

Proteinase inhibitor–containing protein sample
0.1 mg/ml proteinase solution: proteinase for which inhibition is to be assayed, diluted in 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)

1. Run a proteinase inhibitor–containing protein sample on duplicate native polyacrylamide gels as described (see Basic Protocol 3, steps 1 to 2).

   An appropriate, known proteinase inhibitor may be run as a control.

2. Transfer one gel to a tray containing 50 ml of 0.1 mg/ml proteinase solution. Allow the enzyme to diffuse into the gel 30 min at 4°C. Stain second gel with Coomassie brilliant blue as described (see Support Protocol 1, steps 1 to 5).

3. Wash gel briefly with water.

4. Assay for protein-substrate hydrolysis as described (see Basic Protocol 3, steps 3 to 8).

   The presence of an inhibitor appears as a blue band on an otherwise clear background of hydrolyzed casein.

DETERMINATION OF PROTEINASE CLASS

ALTERNATE PROTOCOL 6

Proteinase-containing samples are incubated with a variety of class- or enzyme-specific proteinase inhibitors, separated on a polyacrylamide gel, and activity stained as described in Basic Protocol 3. A clear zone will be evident in lanes where the proteinase is active (i.e., in the absence of inhibitor or in the presence of a mismatched inhibitor). This clear zone will be absent in the lane containing the properly matched proteinase inhibitor, which provides information about the class or type of proteinase detected in the band.

Additional Materials *(also see Basic Protocol 3)*

Proteinase-containing protein sample
Class- or enzyme-specific proteinase inhibitor solution, for example:
200 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol
20 mM Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) in 1 mM HCl
10 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in methanol

1. Mix a proteinase-containing protein sample with 0.1 vol class- or enzyme-specific inhibitor solution. Include samples without inhibitor but with an appropriate amount of solvent used to prepare inhibitors, as well as known proteinases with their inhibitors as controls.
2. Incubate 1 hr at 25°C followed by overnight at 10°C.
3. Assay samples for proteinase activity as described (see Basic Protocol 3, steps 1 to 8).

ESTIMATION OF PROTEIN MOLECULAR WEIGHTS BY SDS-PAGE

SUPPORT PROTOCOL 3

Estimating the molecular weight of a protein is relatively straightforward with SDS-PAGE. Molecular weight standards are separated on a polyacrylamide gel along with the unknown or sample protein, and the migration distance of each is measured. This distance is converted to relative mobility (R_f), which is defined as the distance migrated by a protein divided by the distance migrated by a relative mobility marker. Usually the dye front is chosen as the relative mobility marker. Alternatively, a low-molecular-weight standard protein can be used as this marker. The R_f values of the standards are used to generate a standard curve that is compared to the unknown. The gel concentration should be chosen so that the standards produce a linear curve in the region of the unknown.

Materials

Processed and stained SDS-PAGE gel with molecular weight standards (see Basic Protocol 1; see Alternate Protocols 1 and 3)
Ruler with 0.1-cm markings
Calculator capable of two-variable statistics or computer with spreadsheet or graphing software

1. Measure migration distance of molecular weight standards and unknown protein(s) in a processed and stained SDS-polyacrylamide gel using a ruler with 0.1-cm markings. Measure the distance to the center of the protein band.

   This may be done with a dried gel, a photograph, or a digital image of a gel.

2. Calculate the R_f of each protein band and molecular weight standard by dividing the migration distance by that of the relative mobility marker. Use either the dye front or a low-molecular-weight standard as the relative mobility marker.

   Frequently, with gradient gels an internal protein marker is selected because the dye front has become diffuse or has run off the bottom of the gel.

   In one of the simplest approaches to estimating molecular weights, the migration distance into the gel is used without converting to R_f.

3. Plot R_f (x axis) versus the log[mol. wt.] (y axis) for each molecular weight standard.

   With gradient gels, yet another measurement is frequently plotted on the x axis. By plotting acrylamide percentage (x axis) versus log[mol. wt.] (y axis), very good linearity is obtained. However, adequately straight calibration lines are much more simply determined using migration distance or R_f for the x axis.

4. Use a calculator or computer program to perform linear regression of the plot.

5. Use the equation of the regression line to estimate the size of the unknown protein.

   The purpose of plotting the data and performing the regression is to generate a linear curve through the standards so that the size of the unknown can be estimated. Thus, a region of the plotted data that is reasonably linear should be chosen for performing the regression.

   The general equation of a line is $y = mx + b$, where m is the slope and b is the y intercept. In this case the equation becomes log[mol. wt.] = (slope × R_f) + y intercept.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide solution, 30% (w/v)

Mix 60 g acrylamide (final 30%) and 1.6 g bisacrylamide (final 0.8%) with water in a total volume of 200 ml. Filter the solution through a 0.45-µm filter. Store up to 3 months at 4°C in the dark.

Acrylamide solution, 40% (w/v), high cross-linker

Mix 75.2 g acrylamide (final 40%) and 4.8 g bisacrylamide (final 2.4%) with water in a total volume of 200 ml. Filter the solution through a 0.45-µm filter. Store ≤3 months at 4°C in the dark.

Coomassie brilliant blue staining solution, 0.025% (w/v)

Dissolve 0.5 g Coomassie brilliant blue R250 in 800 ml methanol (final 40%). Add 140 ml acetic acid and water to 2 liters. Store ≤1 month at room temperature.

Coomassie brilliant blue staining solution, 0.1% (w/v)

Dissolve 2 g Coomassie brilliant blue R250 in 800 ml ethanol (final 40%). Add 200 ml acetic acid (final 10%) and water to 2 liters. Store ≤1 month at room temperature.

Denaturing IEF rehydration solution, pH 3 to 10

Dissolve 5.04 g urea (final 8 M) and 788 µl carrier ampholyte mixture (e.g., Pharmalyte; Amersham Pharmacia Biotech; final 7.5%) in water to 10.5 ml. Add a reducing agent (60 mM dithiothreitol) and/or a detergent (0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside), if desired. Make fresh.

Developing solution

Dissolve 25 g sodium carbonate (final 25%) in water to 1 liter. Store ≤2 months at room temperature. Within 1 hr of use, add 20 µl of 37% formaldehyde (final 0.0074%) per 100 ml solution.

Glutaraldehyde fixing solution

4 ml 25% glutaraldehyde (final 0.2%)
150 ml ethanol (final 30%)
13.61 g sodium acetate trihydrate (final 0.2 M)
Water to 500 ml
Prepare immediately before use

Lower (anodic) Tris-tricine tank buffer

Dissolve 121.1 g Tris base (final 0.2 M) and 10 g SDS (final 0.1%) in 500 ml water. Adjust pH to 8.9 with HCl. Dilute to 5 liters. Store ≤1 month at room temperature.

Nonreducing sample buffer, 2×

1.6 ml 4× stacking gel buffer (see recipe)
4 ml 10% (w/v) SDS (final 4%)
1.25 ml glycerol
1.0 mg bromphenol blue
Bring volume to 10.0 ml with water
Store 0.5-ml aliquots ≤ 6 months at –20°C

Running gel buffer, 4×

Dissolve 36.3 g Tris base (final 1.5 M) in 150 ml water. Adjust pH to 8.8 with HCl. Bring volume to 200 ml with water. Store ≤3 months at 4°C.

Running gel overlay

25 ml 4× running gel buffer (see recipe)
1 ml 10% (w/v) SDS (final 0.1%)
Water to 100 ml
Store ≤3 months at 4°C

SDS-PAGE tank buffer, pH 8.3

30.28 g Tris base (final 0.025 M)
144.13 g glycine (final 0.192 M)
10 g SDS (final 0.1%)
Bring to 10 liters with water
Make up directly in large reagent bottles
Store ≤1 month at room temperature

It is not necessary to check pH.

SDS sample buffer, 2×

2.5 ml 4× stacking gel buffer (see recipe)
4.0 ml 10% (w/v) SDS (final 4%)
2.0 ml glycerol (final 20%)
2.0 mg bromphenol blue (final 0.02%)
0.31 g dithiothreitol (DTT; final 0.2 M)
Bring to 10.0 ml with water
Store 0.5-ml aliquots ≤6 months at –20°C

If desired, 5% (v/v) 2-mercaptoethanol may be substitued for DTT. However, DTT is preferred.

Sensitizing solution

300 ml ethanol (final 30%)
68 g sodium acetate (final 6.8%)
2 g sodium thiosulfate (final 0.2%)
Water to 1 liter
Store stock solution ≤2 months at room temperature
Within 1 hr of use, add 0.5 ml of 25% glutaraldehyde (final 0.125%) per 100 ml solution

Silver solution

Dissolve 2.5 g silver nitrate (final 0.25%) in water to 1 liter. Store ≤2 months in a dark bottle at room temperature. Within 1 hr of use, add 40 µl of 37% formaldehyde (final 0.015%) per 100 ml solution.

Stacking gel buffer, 4×

Dissolve 3.0 g Tris base (final 0.5 M) in 40 ml water. Adjust to pH 6.8 with HCl. Bring volume to 50 ml with water. Store ≤3 months at 4°C.

Tris-tricine gel buffer, 4×

Dissolve 72.6 g Tris base (final 3 M) and 0.6 g of SDS (0.3% SDS) in 150 ml water. Adjust pH to 8.45 with HCl. Bring volume to 200 ml with water. Store ≤3 months at 4°C.

Upper (cathodic) Tris-tricine tank buffer

Mix 12.11 g Tris base (final 0.1 M), 17.92 g tricine (final 0.1 M), and 1 g SDS (final 0.1%) with water in a total volume of 1 liter. Do not adjust pH. Store ≤1 month at room temperature.

Water-saturated butanol

Combine 50 ml *n*-, *t*-, or *i*-butanol with 5 ml water. Shake and allow phases to separate. Use top phase (butanol) for overlaying gels. Store indefinitely at room temperature.

COMMENTARY

Background Information

Electrophoresis is the process of moving charged molecules in solution by applying an electrical field across the mixture. Because molecules in an electrical field move with a speed dependent on their charge, shape, and size, electrophoresis has been extensively developed for molecular separations.

As an analytical tool, electrophoresis is simple, relatively rapid, and has unparalleled resolving power. It is used chiefly for analysis and purification of very large molecules such as proteins and nucleic acids. Highly sensitive detection methods have been developed to monitor and analyze electrophoretic separations.

Electrophoresis of macromolecules is normally carried out by applying a sample to a solution stabilized by a porous gel matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. A matrix is required because the electric current passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands in the absence of a stabilizing medium.

The procedures described in this unit all utilize a polyacrylamide gel matrix. The gel forms when a dissolved mixture of acrylamide and bisacrylamide cross-linker monomers polymerizes into long chains that are covalently cross-linked. The gel structure is held together by the cross-linker. Polymerization of acrylamide is a free-radical reaction that is initiated and catalyzed, respectively, by the addition of ammonium persulfate and TEMED.

When the gel solution is poured into a glass-plate sandwich, the top of the solution forms a meniscus. If measures are not taken to prevent this, the gel will polymerize with a curved top, which will cause the separated sample bands to have a similar curved pattern. To eliminate the meniscus, a thin layer of water-saturated butanol is floated on the surface of the gel mixture before it polymerizes. After polymerization, the butanol layer is poured off, leaving the upper surface of the gel flat. The butanol also excludes oxygen, which would otherwise inhibit polymerization on the gel surface.

SDS Polyacrylamide gel electrophoresis

The most widely used method for protein electrophoresis is the SDS-PAGE system of Laemmli (1970); see Basic Protocol 1. This method separates proteins according to their molecular weights. The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 g SDS per gram protein. The bound SDS masks the charge of the proteins themselves, forming anionic complexes with constant net negative charge per unit mass. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds

the protein molecules, minimizing differences in molecular form by eliminating the secondary and tertiary structures. The proteins unfold completely in the presence of a reducing agent such as DTT. The reducing agent cleaves the disulfide bonds, which can form between cysteine residues, and the polypeptides become flexible rods of negative charges with equal charge densities, or charge per unit length. Treating proteins with both SDS and a reducing agent can result in separations exclusively by molecular weight.

There is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle. This linear relationship is valid only for a certain molecular weight range that is determined by the polyacrylamide percentage. The linear separation range can be increased by employing gels cast with a linear gradient of acrylamide percentage (see Alternate Protocol 1). Guidelines for selecting an appropriate acrylamide percentage or gradient for a particular protein molecular weight range are included in Table B3.1.1.

The SDS-PAGE system of Laemmli (1970) utilizes a discontinuous buffer system, meaning that the counter ion of the Tris buffer is different between the tank buffer and the buffer in the gel. The tank buffer contains glycine ions, whose electrophoretic mobility is less than that of the proteins in the sample, and the gel contains chloride ions, whose electrophoretic mobility is higher than that of the proteins in the sample. The sample first passes through a stacking gel of relatively low acrylamide concentration, where the proteins concentrate into a thin zone between the low mobility and the high mobility ion. This stacking effect results in sharp protein bands and exceptional resolution.

This SDS-PAGE system is a modification of a discontinuous system described by Ornstein (1964) and Davis (1964), which was originally devised to separate proteins under nondenaturing (native) conditions (see Alternate Protocol 2). Under native conditions, the migration rate of a protein is dependent on both its intrinsic charge and its size. The molecular weight of the protein therefore cannot be directly determined by its migration in a single gel. The resolution of nondenaturing electrophoresis is generally not as high as SDS-PAGE, but the technique is useful when one wishes to retain the native structure or enzymatic activity of a protein to assay following electrophoresis.

In the SDS-PAGE system of Laemmli, smaller proteins comigrate with SDS micelles. This prevents the separation of proteins smaller than ~10 kDa. A modified buffer system described by Schägger and von Jagow (1987) was developed to allow separations of peptides and smaller proteins. A modification of this technique (see Alternate Protocol 3) incorporates 35% (v/v) ethylene glycol and a high proportion of bisacrylamide cross-linker (6% of the total monomer) in the running gel to further optimize separation of small peptides.

In the discontinuous systems described in this unit (see Basic Protocol 1; see Alternate Protocols 1, 2, and 3), the choice between a standard gel and a mini-format gel must be made. Mini-format gels are now more commonly run than standard gels due to the higher speed with which they can be run and the increased ease of handling smaller gels. Standard gels are used when higher resolution or greater separation distance is required.

Isoelectric focusing

IEF is an electrophoretic method that separates proteins according to their pIs. Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino and carboxyl termini. The pI is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI, and negatively charged at pH values above their pI.

The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. A protein with a positive net charge will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will migrate toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the *focusing* effect of IEF, which concentrates proteins at their pI and allows proteins to be separated on the basis of very small charge differences.

The degree of resolution is determined by electric field strength. IEF is therefore performed at high voltages (typically >1000 V). When the proteins have reached their final

positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically <1 mA).

The methods for IEF described in this unit (see Basic Protocol 2; see Alternate Protocol 4) are dependent on a carrier ampholyte–generated pH gradient. Carrier ampholytes are small, soluble, amphoteric molecules with a high buffering capacity near their pI. Commercial carrier ampholyte mixtures are comprised of hundreds of individual polymeric species with pIs spanning a specific pH range. When a voltage is applied across a carrier ampholyte mixture, the carrier ampholytes with the lowest pI (and the most negative charge) move toward the anode, and the carrier ampholytes with the highest pI (and the most positive charge) move toward the cathode. The other carrier ampholytes align themselves between the extremes, according to their pIs, and buffer their environment to the corresponding pHs. The result is a continuous pH gradient.

IEF can be run in either a native or denaturing mode. Native IEF is the more convenient option, as precast native IEF gels are available in a variety of pH gradients. This method is also preferred when native protein is required, as when activity staining is to be employed. The use of native IEF, however, is often limited by the fact that many proteins are not soluble in water at low ionic strength, or have low solubility close to their isoelectric point. In these cases, denaturing IEF is employed. Urea is the denaturant of choice, as this reagent can solubilize many proteins not otherwise soluble under IEF conditions. Detergents and reducing agents are often used in conjunction with urea for more complete unfolding and solubilization. Urea is not stable in aqueous solution, so precast IEF gels are not manufactured with urea. Rehydrating a dried gel with urea, carrier ampholytes, and other additives is a convenient alternative.

Protein visualization

After an electrophoresis run is complete, the gel must be analyzed to answer analytical or experimental questions. As most proteins are not directly visible, the gel must be processed to determine the location and amount of the separated proteins. The most common analytical procedure is staining. Proteins are usually stained with Coomassie brilliant blue or silver nitrate.

Coomassie brilliant blue staining (see Support Protocol 1) is based on the binding of the dye Coomassie brilliant blue R250, which binds nonspecifically to virtually all proteins. The gel is impregnated with a solution of the dye. Dye that is not bound to protein is allowed to diffuse out of the gel during the destain steps. Although Coomassie brilliant blue staining is less sensitive than silver staining, it is widely used due to its convenience. Coomassie brilliant blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

When staining IEF gels with Coomassie brilliant blue, the gel is first fixed in a trichloroacetic acid solution to leach out the carrier ampholytes, which would otherwise cause background staining. When staining small peptides run on Tris-tricine gels, the gel is first fixed in a solution containing glutaraldehyde, which cross-links the peptides and prevents them from diffusing out of the gel during subsequent staining steps.

Silver staining is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels. This sensitivity, however, is obtained at the expense of high susceptibility to interference from a number of factors. Precise timing, high quality reagents, and cleanliness are essential for reproducible, high-quality results. In silver staining, the gel is impregnated with soluble silver ion and developed by treatment with formaldehyde, which reduces silver ion to metallic silver, which is insoluble and visible. This reduction is promoted in the presence of proteins, leading to visible bands on the gel. There are many variations of the silver staining process. The method described (see Support Protocol 2) is based on the method of Heukeshoven and Dernick (1985) and has been selected for its overall convenience, sensitivity, reproducibility, and speed.

Activity staining

Techniques have also been developed for the specific visualization of particular classes of enzymes following electrophoretic separation in a gel. These techniques are often referred to as "activity staining," as the intrinsic activity of the enzyme is used, either to produce a colored product or to produce a clear zone on a colored background within the gel. A method for visualizing proteinases based on the work of García-Carreño and Haard (1993) and García-Carreño et al. (1993) is presented (see Basic Protocol 3).

Following electrophoresis of samples that are not heat treated, the gel is impregnated with a good general protein substrate (casein). Pro-

teinases within the gel are allowed to digest the casein, and the gel is stained with Coomassie brilliant blue. The casein in the gel produces a uniform blue background stain, except where it has been digested by proteinases in the gel to produce small peptides that diffuse out of the matrix. Proteinases are thus visualized as clear bands against a blue background.

This technique can be applied following SDS-PAGE, as most proteinases are monomeric, and hence minimally affected by SDS (García-Carreño et al., 1993). The technique described is applicable to most proteinases, which are active at neutral to alkaline pH. A method for characterizing proteinase inhibitors (see Alternate Protocol 5), a growing are of study in food science (García-Carreño, 1996; García-Carreño and Hernandez-Cortes, 2000; García-Carreño et al., 2000) is also included. Recently, a separation technique for acid proteinases with high pIs was published (Díaz-López et al., 1998).

Critical Parameters and Troubleshooting

Many factors influence the quality of electrophoretic separations, including gel preparation, reagent quality, instrument assembly, electrophoresis conditions, and the nature and quantity of the sample.

Preparing and running the gel

To have a high-quality gel, acrylamide polymerization must be complete and uniform. Polymerization may be inhibited by low temperatures, oxygen, insufficient or degraded catalyst, and low acrylamide concentrations. Any of these factors can prevent complete polymerization or, in extreme cases, prevent polymerization entirely. Insufficient polymerization results in poorly defined sample wells and distorted bands. Air bubbles trapped under the comb teeth can inhibit polymerization locally, also resulting in band distortions. Insufficient polymerization along spacers can cause the gel to run faster towards the edges, producing a localized "frown" effect. Polymerization may also be uneven across the entire gel, resulting in distortions in the final electrophoresis result.

The following suggestions should minimize problems of incomplete polymerization. Warm all refrigerated gel solutions to room temperature prior to use and always deaerate the gel solution 5 to 10 min with at least a water aspirator. It is helpful to warm the gel solution to 20° to 25°C after deaeration and allow it to polymerize at or slightly above room temperature. Check the ammonium persulfate for freshness. Fresh ammonium persulfate will crackle when water is added; if it doesn't, use a fresh bottle. Check the freshness of the acrylamide stock solution. Old acrylamide can also inhibit polymerization. If the polymerization problem persists, increase the ammonium persulfate and TEMED concentrations by 50%. Increasing the catalyst concentration is particularly useful when working with low acrylamide concentrations.

Too rapid polymerization can result from high temperatures or too much catalyst. If polymerization is too rapid, the gel may not polymerize evenly, or may polymerize before the gel is completely poured. To prevent these problems, decrease the amount of ammonium persulfate and TEMED by 30%.

Reagents used in preparing electrophoresis gels and buffers must be of high quality. Many reagent manufacturers supply specifically designated "electrophoresis quality" reagents that are prepared to be largely free of interfering contaminants. Use such reagents whenever possible. Also take care when preparing the buffers and other solutions used in electrophoresis. Mistakes in concentration or pH adjustment can result in a slowly running gel or a low-quality electrophoretic separation.

Many electrophoresis problems can be attributed to improper instrument assembly. Sealing gaskets must be seated correctly and spacers must be aligned or buffer leakage may result. If the level of upper buffer drops too far, electrical continuity is interrupted and protein migration is stopped. Air bubbles trapped between the glass plates at the bottom of the gel cassette can be large enough to block current locally and cause a distortion in the gel pattern. Care should be taken to make sure the bottom of the gel cassette is free of bubbles.

Electrophoresis generates heat, and if the gel runs faster than this heat can dissipate, it will not run evenly. Running the gel too fast is the primary cause of "smiling," where the center of the gel runs faster than the edges. The use of an electrophoresis unit that allows the gel cassette to be completely submerged in the lower buffer allows more efficient dissipation of heat and can allow the gel to run faster without smiling. The use of external cooling (connecting the electrophoresis unit to a thermostatic circulator) results in still more effective dissipation of heat.

Preparing the sample

To be analyzed effectively by electrophoresis, a protein sample must be well solubilized, undegraded, free of particulate material, and loaded at an appropriate concentration. Poor solubilization is manifested by vertical streaks or smears rather than distinct bands. In the case of SDS-PAGE, heating briefly in SDS-containing treatment buffer is usually sufficient to solubilize all of the proteins in the sample. Some proteins, however, aggregate during heating to 100°C. In these cases, it is better to solubilize at lower temperatures (40° to 80°C).

Many proteins are poorly soluble under conditions prevailing in nondenaturing electrophoresis or IEF. Solubility can occasionally be improved with the use of a nondenaturing, neutral detergent (e.g., 0.5% [w/v] Triton X-100, CHAPS, or octyl glucoside) or polyalcohol (e.g., 20% [w/v] glycerol or sorbitol) in both the sample and the gel.

Degradation by endogenous proteinases can result in smearing, loss of high-molecular-weight proteins, or loss or splitting of bands. Most proteinases are inactivated by heating in the presence of SDS, so if SDS-PAGE is being employed, the sample should be heated as soon as possible. Up until the heating step (if one is employed) samples should be kept on ice to slow proteolysis. If proteolysis remains a problem, proteinase inhibitors (e.g., phenylmethylsulfonyl fluoride, leupeptin, pepstatin) should be used during sample preparation.

Protein can be prevented from entering the gel cleanly by particulate and unsolubilized material, which will remain in the sample wells. Samples containing particulate material should therefore always be centrifuged prior to electrophoresis.

When too much protein is loaded onto a gel, bands may spread and not resolve well. The staining may saturate, producing indistinct bands. Too little protein will result in bands of interest being faint or absent. The optimal amount to load varies widely depending on the complexity of the sample and the method of staining. It is often useful to load several different dilutions of a sample on the same gel to determine the optimal amount.

Whereas SDS-PAGE and other discontinuous techniques are generally quite tolerant of sample impurities and buffer and ionic variations, the quality of the sample and the nature of the solution it is loaded in have a strong influence on the quality of an IEF separation. The sample must be as free as possible of salts, buffers, and other small charged molecules, otherwise the proteins will not focus into sharp bands.

Staining the gel

Coomassie brilliant blue staining, although approximately 50-fold less sensitive that silver staining, is considerably simpler to perform and less subject to interference or error than silver staining. Insufficient staining intensity may be the result of not staining long enough, in which case, the gel should be left longer in the staining solution. The blue background should be removed by destaining until the background is clear.

Silver staining, on the other hand, is highly susceptible to interference from a variety of sources. Exceptional cleanliness must be practiced in preparing the electrophoresis unit and in handling the gel. All equipment used for running and staining the gel must be cleaned with detergent and thoroughly rinsed. Clean gloves should be worn when handling the electrophoresis apparatus, the gel, or the staining tray. High-quality water must be used to prepare the reagents, as impurities have a strong effect on silver staining. For best results, water with a resistivity ≥5 MΩ should be used. The reagents used for silver staining should be of the highest quality possible.

Temperature has a strong effect on silver staining, with higher temperatures promoting faster development and darker background. For optimal reproducibility, the timing of silver staining steps should be precisely controlled. The timing of the wash steps is particularly important. Faint bands or poor development could be the result of poor reagent quality or improperly prepared reagents. It could also be the result of washing too long between the silver step and the developing step. An excessively dark background could also be the result of poor reagent quality or improperly prepared reagents. It may also be the result of developing too long, the use of impure water or detergent, or the presence of other residues in the staining tray.

Proteinases and proteinase inhibitors in a sample should be analyzed for their sensitivity to SDS concentration before activity staining is carried out. Their activity should be measured in the presence and absence of 0.1% SDS before proceeding with electrophoresis. The author has characterized many proteinases under such conditions without a loss of activity. Most SDS-sensitive enzymes regain their activity if the gel is washed in the buffer used to dissolve the

substrate before proceeding with the activity staining.

Anticipated Results

Properly executed, SDS-PAGE should be able to resolve a protein sample into up to 100 distinct protein species. Proteins separated by nondenaturing PAGE appear more diffuse and exhibit less overall resolution than proteins separated under denaturing conditions; however, biological activity is often maintained. Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins; however, they are more difficult to prepare. Molecular weight calculations are simplified with gradient gels because of the extended linear relationship between size and protein position within the gel.

IEF has a similar resolving power to SDS-PAGE, but it has less applicability due to the limited solubility of many proteins under IEF conditions.

With Coomassie brilliant blue staining one should be able to detect ~50 to 100 ng protein in a normal band. The lower detection limit of silver staining is ~1 ng protein. Once the gel is stained, it can be photographed or dried on a transparent backing for a record of the position and intensity of each band.

Time Considerations

Preparation of electrophoresis running and stacking gels requires 2 to 3 hr. Running the gel requires 1.5 hr for mini-format gels or IEF gels and 5 hr for standard gels. Visualization of the electrophoresis result takes a full day for Coomassie brilliant blue staining and 3 hr for silver staining. Activity staining a gel for proteinase requires ≥6 hr (including the destaining solution wash) after running the polyacrylamide gel. The determination of proteinase class requires an additional overnight incubation before the gel can be run.

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Good general references on protein electrophoresis that contain detailed descriptions of several important techniques.

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Electroblotting from Polyacrylamide Gels

Electroblotting of proteins from polyacrylamide gels onto retentive membranes is usually performed to facilitate procedures leading to protein identification and characterization. It has been used for immunoblotting (or Western blotting), N-terminal protein sequencing, in situ protease or chemical cleavage of proteins for further structural analysis, protein band detection using sensitive stains such as AuroDye colloidal gold, and protein elution. The electroblotting methods in this unit require that the proteins have already been separated on a polyacrylamide gel (*UNIT B3.1*). However, if the blotted protein bands will be used for N-terminal protein sequencing or sequencing of cleavage/digestion products, refer to the specific requirements detailed in Alternate Protocol 1, which may affect electrophoresis conditions. Including protein markers or prestained standards in gels is useful because they will transfer to the membrane and can be used to indicate the size of proteins after immunostaining.

This unit contains procedures for electrophoretically transferring proteins onto a variety of membranes including polyvinylidene difluoride (PVDF; Basic Protocol 1) and nitrocellulose (Alternate Protocol 2) and derivatized membranes. The choice of membrane type for electrotransfer is dependent on the ultimate application for the blot membrane. High-retention PVDF binds proteins tightly and is well suited for applications such as sequencing where high protein amount is critical (Alternate Protocol 1), whereas low-retention membranes may be advantageous for subsequent protein extraction or immunoblot analysis (*UNIT B3.4*). Most protocols are designed for use with tank transfer setups; Alternate Protocol 3 presents procedures for electroblotting in semidry systems.

In some cases, stained blots are used only to identify protein band patterns while leaving the gel unmodified for subsequent steps (*UNIT B3.3*). If such minimal protein transfer is desired, contact blotting is a suitable alternative. This unit also describes procedures for eluting proteins from membranes using detergents (Basic Protocol 2) or acidic extraction with organic solvents (Alternate Protocol 4).

Unless otherwise indicated, the following protocols are primarily designed for electrotransferring proteins from SDS gels. As proteins in other types of gels have much lower charge densities and, sometimes, opposing net charges for different proteins, substantial modifications to the protocols must be made for these specialized applications. Additional procedures for electrophoresis and blotting of proteins can be found in Coligan et al. (2001).

ELECTROBLOTTING ONTO PVDF MEMBRANES

BASIC PROTOCOL 1

The following procedure is based on electrotransfer from polyacrylamide gels containing 0.2% sodium dodecyl sulfate (SDS) in the gel solution and electrophoresis and sample buffers. It uses a Bio-Rad solid plate tank transfer apparatus with 7 cm spacing between the electrode plates, which can accommodate gels up to 14 × 18 cm in size and requires ~2.5 liters transfer buffer. The choice of PVDF membrane depends on the planned subsequent use: high-retention Trans-Blot membranes (Bio-Rad) are suitable for protein sequencing, whereas low-retention Immobilon-P membranes (Millipore) are recommended for low-background immunodetection or staining and for recovering proteins from membranes.

Contributed by Sandra Harper and David W. Speicher
Current Protocols in Food Analytical Chemistry (2001) B3.2.1-B3.2.14

Materials

1× transfer buffer (see recipe)
Polyacrylamide gel containing proteins of interest (*UNIT B3.1)*
100% methanol

Powder-free gloves
Electroblotting apparatus: "solid" plate electrode tank transfer system (e.g., Trans-Blot Cell, Bio-Rad)
Glass dishes and trays
Gel support sheet (e.g., porous polyethylene sheet, Curtin Matheson)
PVDF transfer membrane: 0.2 µm PVDF membrane (Bio-Rad) or Immobilon-P (Millipore)
Whatman no. 1 filter paper
Power supply (500 V, 300 mA)

Additional reagents and equipment for staining gels (*UNIT B3.1*)

NOTE: Use powder-free gloves when handling all materials for this procedure and handle membranes with forceps at the edges to avoid potential artifactual staining.

Equilibrate the gel and membrane in transfer buffer

1. Prior to blotting, prepare the blotting apparatus by thoroughly rinsing with high-purity water.

   The optional porous polyethylene sheet is hydrophobic, and it is difficult to get water or buffer into the pores. Be sure to hydrate or "wet" it properly by spraying water under pressure through the sheet or by submerging the entire polyethylene sheet in methanol and then submerging in high-purity water.

2. Fill the transfer tank with 1× transfer buffer. Submerge the gel cassette holder, fiber pads, and polyethylene sheet (after initial hydration as described in step 1) in transfer buffer. Place them inside the tank or submerge in transfer buffer using a separate tray.

   The transfer buffer can be left in a covered transfer tank for up to 2 hr prior to use. Longer times should be avoided because the methanol in the buffer will evaporate, significantly changing the buffer composition.

3. Prepare the polyacrylamide gel containing proteins of interest by disassembling the gel apparatus, removing the stacking gel with a razor blade, and cutting a small piece from the lower left-hand corner of the gel (near lane 1 for gels loaded left to right; see Fig. B3.2.1) to aid in identifying the lanes in subsequent steps.

4. Briefly equilibrate the gel in a glass dish containing ~250 ml of 1× transfer buffer for an appropriate time based on gel thickness (0.5-mm-thick gels for 1 min; 0.75- to 1-mm gels for 5 min; 1.5-mm gels for 15 min).

   Longer equilibrations in transfer buffer prior to electrotransfer may extract too much SDS from the gel and hence reduce transfer efficiency. This equilibration step will remove excess SDS and prevent the gel from swelling during transfer. The negatively charged SDS allows proteins to move toward the transfer membrane in an electric field. On the other hand, too much SDS decreases binding of protein to PVDF membranes. Therefore, the amount of SDS is critical for transfer yield (see Critical Parameters and Troubleshooting).

5. Cut the PVDF transfer membrane about 0.5 to 1 cm larger than the gel. Cut a piece of Whatman no. 1 filter paper ~0.5 cm larger than the PVDF membrane.

6. Wet the PVDF membrane in a clean glass dish containing 100% methanol for 5 sec. Transfer membrane to a tray containing transfer buffer.

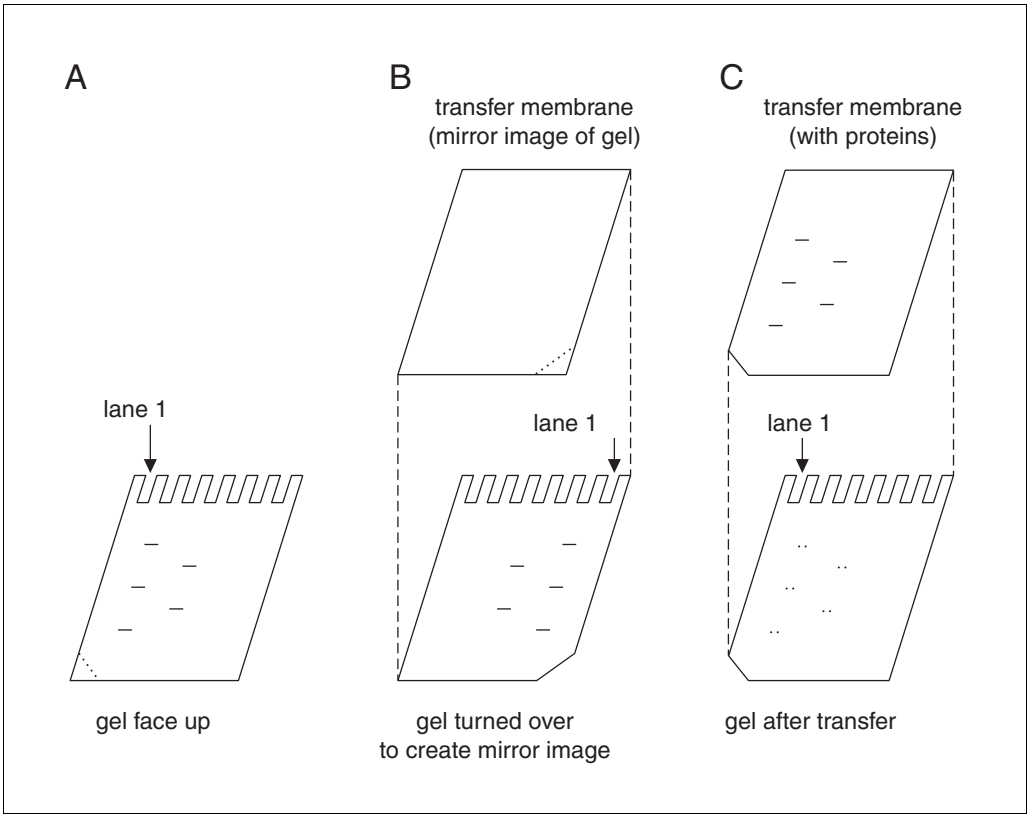

Figure B3.2.1 Aligning the gel for transfer. (**A**) If the polyacrylamide gel has been loaded from left to right, cut a small piece from the corner of the lower left-hand edge of the gel near the first lane. (**B**) When preparing the transfer sandwich, turn the gel over so that the cut edge is on the lower right-hand corner of the gel. This will ensure that the transferred proteins will appear in the same order as in the original gel. (**C**) After transfer, trim the membrane above the cut corner of the gel to mark orientation. Dots indicate where the proteins were before transfer.

The PVDF membrane will wet almost immediately with the 100% methanol. The hydrophobic membrane will not wet in either water or transfer buffer alone. After initial wetting in methanol, do not let the membrane dry; if drying occurs, rewet the membrane in 100% methanol.

Prepare the gel/membrane transfer sandwich

7. Place the opened gel holder cassette on a clean, flat surface. Place one transfer buffer–wetted fiber pad (see step 2) on the top surface, followed by the gel support sheet.

   A sheet of porous polyethylene is preferred as a support because it is rigid and facilitates handling, but filter paper can be used as a simple alternative. If the polyethylene sheet is used, place it with the smooth side up (toward the gel).

8. Place the gel face down on the support, so that the cut edge is now on the right-hand side (see Fig. B3.2.1B). Pour 5 to 10 ml transfer buffer on top of the gel.

9. Remove wet PVDF membrane from the tray containing transfer buffer (step 6). Eliminate air bubbles on both surfaces of membrane by sliding the membrane across the edge of the glass tray. Resubmerge membrane in transfer buffer for a few seconds and then position membrane above gel, letting its center contact the gel, and slowly lower the membrane from the center outward to force any bubbles to the edge of the gel. Rinse gloved hands with high-purity water and smooth the membrane gently to

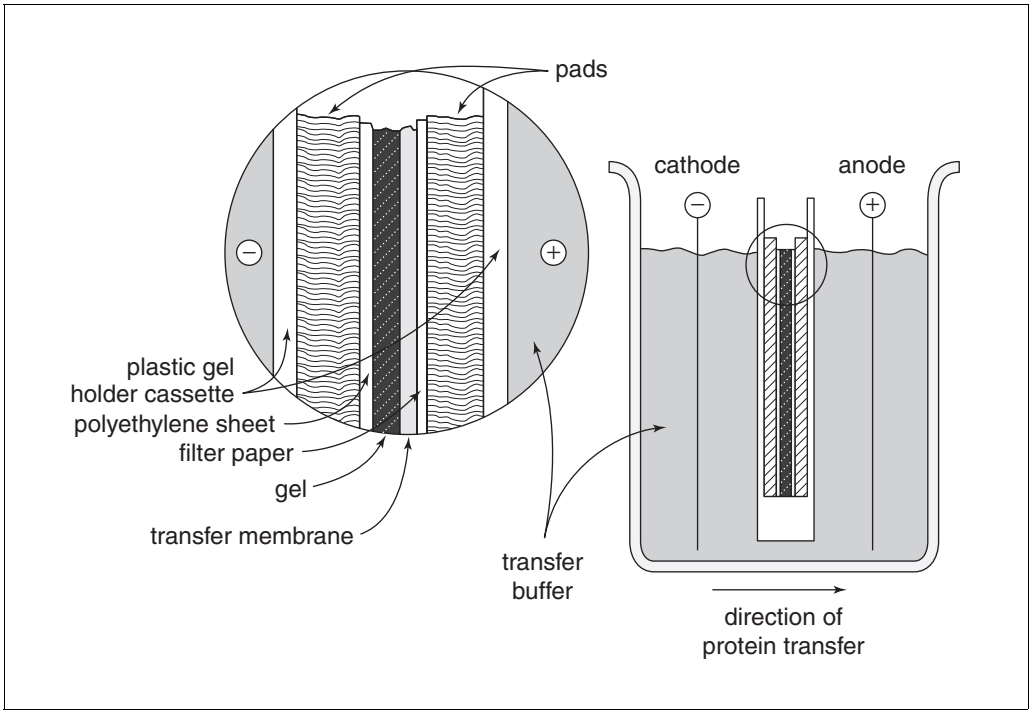

Figure B3.2.2 Electroblotting with a tank transfer unit. The polyacrylamide gel containing the protein(s) to be transferred is placed on the smooth side of the polyethylene sheet (or filter paper sheets) and covered with the PVDF membrane and then a single sheet of filter paper. This stack is sandwiched between two fiber pads and secured in the plastic gel holder cassette. The assembled cassette is then placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane.

ensure uniform contact with the gel. Inspect the membrane for any trapped air bubbles.

This step is extremely important. Air bubbles will prevent the transfer of proteins.

10. Briefly wet the filter paper with transfer buffer and cover the PVDF membrane. Smooth gently to remove any air bubbles. Place the other fiber pad on top of the membrane and close the holder.

Do not allow any area of the PVDF membrane to dry during assembly, or transfer will not occur in these areas. The transfer sandwich should fit together snugly to provide good contact between the membrane and gel. The complete gel sandwich should look like that in Figure B3.2.2.

Conduct protein electrotransfer

11. Slide the assembled transfer cassette into the tank with the gel on the cathode side and the membrane on the anode side.

Proteins in an SDS-polyacrylamide gel have a net negative charge and will migrate toward the anode. For transfer from another type of gel, the orientation of the cassette may need to be reversed.

12. Fill the transfer tank with 1× transfer buffer so that the buffer completely covers the electrode panels but does not touch the electrical connectors.

13. Connect the power supply and perform the transfer at constant current compatible with complete transfer from the gel. For a Bio-Rad Trans-Blot tank transfer apparatus

with solid plate electrodes, use 200 to 250 mA constant current. Transfer 0.5-mm gels for 1 hr, 0.75- to 1-mm gels for 2 hr, and 1.5-mm gels for 3 hr.

Longer times do not appear to increase transfer yield but may lead to overheating of the gel. Transfers can be carried out at constant voltage as well, but the current may increase during the run. Use caution to prevent overheating and/or unacceptably high current. Because of the amount of heat generated at higher transfer rates, it may be necessary to run the transfer in a cold room or with a cooling core to prevent overheating.

14. At the end of the transfer period, turn off the power and disconnect the power supply. Open the transfer sandwich and remove the membrane and gel (Fig. B3.2.1). Thoroughly rinse the membrane with high-purity water, three times, 5 min each.

The membrane can be stained as described in UNIT B3.3 or used for other detection methods. Stained or unstained membranes can be air dried (for ~30 min) and stored in resealable plastic bags at room temperature for several weeks or at −20°C for a permanent record. Dried membranes can be rehydrated in 50% or 100% methanol and used for subsequent detection procedures.

15. To assess the efficiency of transfer, stain the proteins remaining on the gel with Coomassie blue or with more sensitive silver solutions (*UNIT B3.1*), depending on the amount of protein originally loaded on the gel.

ELECTROBLOTTING OF PROTEINS FOR SEQUENCE ANALYSIS

ALTERNATE PROTOCOL 1

One-dimensional or two-dimensional gel electrophoresis combined with electroblotting to PVDF membranes provides a method to obtain proteins of high purity that can be used directly for N-terminal protein sequence analysis. The major concern is avoiding possible chemical modification of proteins such as N-terminal blocking. Simple precautions such as casting gels in advance and modifying the composition of reagents to protect proteins minimize the possibility of side reactions during electrophoresis and electroblotting and are described in this protocol.

NOTE: Use high-quality water such as Milli-Q-purified water or equivalent and high-purity electrophoresis reagents (i.e., Bio-Rad) throughout for best results.

Additional Materials *(also see Basic Protocol 1)*

Thioglycolate (thioglycolic acid, sodium salt; Sigma)
PVDF transfer membrane: 0.2-µm PVDF membrane (Bio-Rad), ProBlott (Perkin-Elmer), or Immobilon-P^{SQ} (Millipore)

Additional reagents and equipment for one- or two-dimensional gel electrophoresis (e.g., *UNIT B3.1*) and for staining membranes (*UNIT B3.3*)

1. Cast polyacrylamide gel including stacking gel at least 24 hr but not more than 48 hr prior to use. Store the gel at room temperature until needed, being sure to protect it from dehydration (e.g., store submerged in high-purity water).

For preparing gels choose an acrylamide concentration such that the protein will migrate as a sharp, tight band with an R_f between 0.2 and 0.8. Casting a gel in advance allows complete polymerization, reduces the amount of oxidants and free radicals, and minimizes the possibility of blocking the N terminus or other amino groups of the protein.

2. Add thioglycolate to the electrophoresis buffer in the cathode buffer chamber to a final concentration of 0.1 mM (11.4 mg per liter buffer).

Thioglycolate scavenges remaining free radicals and oxidants in the gel.

3. Solubilize samples in either 2× SDS sample buffer containing sucrose or glycerol but not urea. Heat to 37°C for 15 min.

If possible, avoid boiling the sample, as the high temperature increases the risk of chemical modification of the protein. Boiling may be necessary for complete solubilization of some samples, however (e.g., whole viruses).

DTT or 2-ME in the sample buffer is acceptable.

4. Conduct gel electrophoresis. Proceed with electroblotting (see Basic Protocol 1, steps 1 to 14).
5. After transfer is complete, rinse the membrane six times for 5 min each with a large volume of water (at least 200 ml each time).

   The membrane must be thoroughly rinsed with water immediately after transfer is complete. It is critical that the membrane is not allowed to dry prior to thorough rinsing because even partial drying can prevent effective removal of Tris and glycine.

6. Stain the membrane with amido black or Ponceau S (*UNIT B3.3*) to detect proteins. Stain the gel (*UNIT B3.1*) after transfer as appropriate to judge transfer efficiency.

ALTERNATE PROTOCOL 2

ELECTROBLOTTING ONTO NITROCELLULOSE MEMBRANES

The electroblotting procedure for nitrocellulose membranes differs little from that for PVDF membranes. Nitrocellulose is compatible for use with a moderate amount of SDS (up to 0.1%) in the transfer buffer. The delicate membranes must, however, be handled carefully and protected from high concentrations of organic solvents.

Additional Materials *(also see Basic Protocol 1)*

0.45-µm nitrocellulose transfer membrane (Schleicher & Schuell)

Additional reagents and equipment for staining membranes (*UNIT B3.3*)

1. Prepare the transfer apparatus and gel as described for PVDF membranes (see Basic Protocol 1, steps 1 to 4).
2. Cut the nitrocellulose transfer membrane to a size slightly larger than the gel and cut a piece of filter paper slightly larger than the membrane.
3. Wet the membrane by slowly introducing the membrane from one corner into a glass dish of 1× transfer buffer. Equilibrate the membrane for 15 min.

   IMPORTANT NOTE: *Never soak the membrane in 100% methanol. During electrotransfer and staining, avoid exposure to high concentrations of organic solvents, which will dissolve the membrane. Up to 20% methanol can be used in the transfer buffer without affecting the membrane.*

4. Proceed with electrotransfer as above for PVDF membranes (see Basic Protocol 1, steps 7 to 13).
5. When transfer is complete, rinse membrane three times for 5 min each with high-purity water; detect proteins on the membrane using an aqueous stain such as Ponceau S (*UNIT B3.3*) if desired.
6. Assess efficiency of transfer by staining the gel (*UNIT B3.1*) after transfer as appropriate.

PROTEIN ELECTROBLOTTING IN SEMIDRY SYSTEMS

An alternative to the tank transfer system is the semidry transfer system. In this procedure, the gel is stacked horizontally on top of the membrane in the transfer apparatus. Because only a small volume of transfer buffer is used, SDS from the gel is less effectively diluted, which may result in incomplete binding and lower yields, especially with PVDF membranes. For this reason, semidry transfer units are not recommended when reproducible high recoveries of electroblotted proteins are desired (e.g., for subsequent sequence analysis). Some procedures recommend stacking multiple transfer sandwiches to achieve several transfers simultaneously. To prevent unbound protein from migrating through the next gel and onto the membrane in the next transfer stack, sheets of porous cellophane sheets or dialysis membrane are placed between adjacent transfer stacks (see Fig. B3.2.3). Semidry electrotransfer requires shorter transfer times than tank transfer.

Additional Materials *(also see Basic Protocol 1)*

Semidry transfer apparatus
Mylar mask (optional)

Prepare the transfer sandwiches

1. Cut transfer membrane just slightly larger than the gel containing proteins to be transferred, then equilibrate in the appropriate transfer buffer (see Basic Protocol 1, step 6).

2. Cut six sheets of filter paper the same size as the transfer membrane and wet thoroughly in transfer buffer.

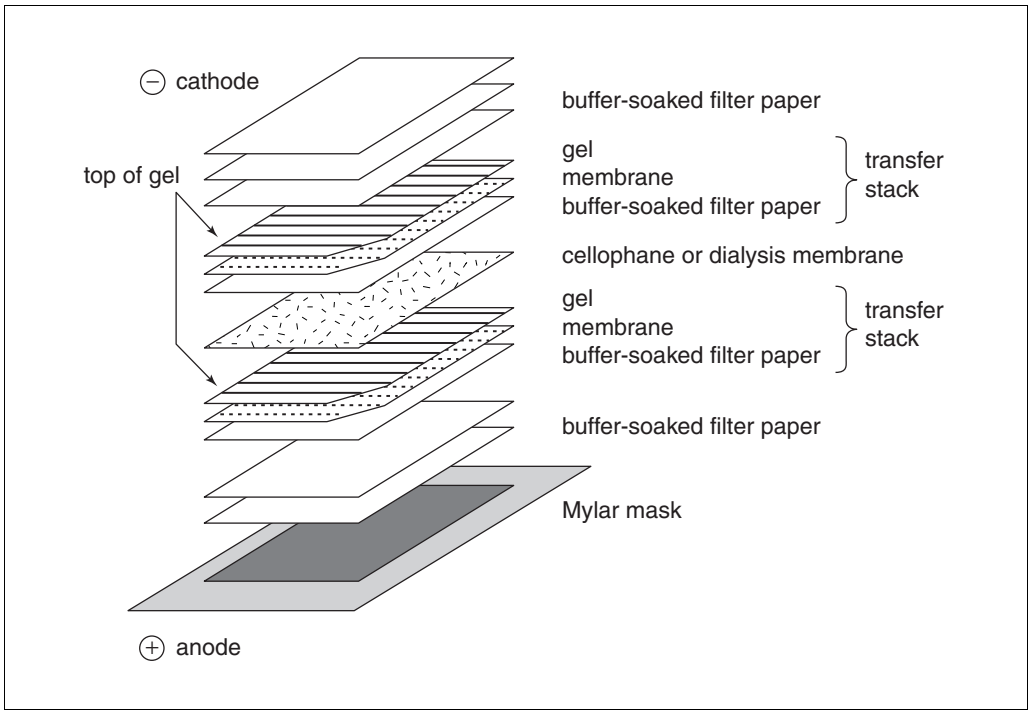

Figure B3.2.3 Electroblotting with a semidry transfer unit. In most cases, the lower electrode is the anode, as shown. Position the Mylar mask (optional) directly over the anode. Layer on three sheets of filter paper that have been wetted in transfer buffer. For negatively charged proteins, place the preequilibrated transfer membrane on top of the filter paper followed by the gel and three additional sheets of wetted filter paper. If multiple gels are to be transferred, separate the transfer sandwiches by inserting a sheet of porous cellophane or dialysis membrane between each stack. Place the cathode on top of the assembled transfer stack(s). Transfer the proteins by applying a maximum current of 0.8 mA/cm^2 gel area.

3. Remove gel containing proteins of interest from the electrophoresis unit. Using a razor blade, cut the lower corner of the gel at the first lane. Equilibrate the gel in transfer buffer (see Basic Protocol 1, step 4).

4. Layer a Mylar mask (optional) followed by three sheets of filter paper on the anode of the transfer unit. Smooth each piece of filter paper to avoid trapping air bubbles between the layers.

   It is important to remove all air bubbles from the transfer stack as they will block the flow of current through that area of the gel, creating blank spots on the transfer membrane.

5. Layer the prepared transfer membrane on top of the filter paper. Gently roll a test tube or Pasteur pipet over the membrane to push out any air bubbles.

6. Place the gel on top of the transfer membrane so that the cut edge is on the left-hand side. With a pair of scissors, cut the lower corner of the membrane even with the gel (to aid in realigning gel and blot after final staining). Again, remove any air bubbles (see Basic Protocol 1, step 9).

   When assembling this transfer sandwich, the gel is placed on top of the membrane, in contrast to the opposite order for tank type sandwich assembly as in Basic Protocol 1. Therefore, it is unnecessary to turn the gel over to obtain the proper orientation (compare Figs. B3.2.1 and B3.2.3).

7. Layer the remaining three sheets of filter paper individually on top of the gel, rolling out any air bubbles after each addition.

   It is possible to transfer multiple gels simultaneously using semidry blotting. As shown in Figure B3.2.3, a sheet of porous cellophane (Hoefer Pharmacia) or dialysis membrane (Bio-Rad or Sartorius), preequilibrated for 5 min in transfer buffer, can be placed between the transfer stacks to prevent proteins from migrating onto an adjacent transfer stack membrane. However, proteins on the gel closest to the anode tend to be transferred more efficiently; thus, transferring multiple gels simultaneously is not recommended for critical applications such as protein sequencing.

Perform the protein electrotransfer

8. Attach the cathode unit on top of the transfer sandwich and connect leads to the power supply. Transfer proteins at constant current for no more than 1 hr.

   Do not exceed 0.8 mA/cm^2 gel surface area. If the outside of the unit becomes warm during transfer, the current is too high and should be lowered.

9. After the transfer period, turn off the power supply and disconnect the leads. Remove the cathode and uppermost three layers of filter paper. Cut the corner of the transfer membrane above the cut corner of the gel. Alternatively, mark the transfer membrane by tracing the gel with a soft lead pencil.

10. Proceed with protein staining or immunodetection, as desired, or dry and store membranes at −20°C for later use (optional).

PROTEIN ELUTION FROM PVDF MEMBRANES USING DETERGENTS

Binding affinities of most proteins to PVDF membranes are relatively strong. The most efficient protocol for protein recovery from PVDF membranes requires the use of detergents, which limits the possible use of extracted samples because detergents are often incompatible with subsequent procedures. This protocol is a simple procedure to elute proteins from the membrane into a Triton/SDS solution. A protocol that employs acidic extraction with organic solvents is also described (Alternate Protocol 4).

Materials

Polyacrylamide gel containing proteins of interest
Ponceau S stain (*UNIT B3.3*; optional)
50% methanol (optional)
Triton/SDS elution buffer (see recipe)

PVDF transfer membrane (Immobilon-P, Millipore)
Transfer apparatus: solid plate electrode tank transfer system (e.g., Trans-Blot, Bio-Rad)

Additional reagents and equipment for staining membranes (*UNIT B3.3*)

1. Prepare gel and membrane for transfer as for PVDF membranes (see Basic Protocol 1, steps 1 to 6).
2. Assemble the gel/membrane transfer sandwich and place in transfer tank (see Basic Protocol 1, steps 7 to 10).
3. Conduct electrotransfer (see Basic Protocol 1, steps 11 to 14), using lower field strengths for step 13 (e.g., transfer for 6 hr at 100 mA for 1.5-mm gels).

   Temperatures >20°C during the transfer can increase the interaction between protein and matrix and make extraction more difficult. If problems arise in eluting proteins from the membrane, try running the transfer in a cold room overnight at low currents (50 mA).

4a. Visualize transferred proteins with any membrane-compatible stain (*UNIT B3.3*). If bound stain will interfere with subsequent steps, use one of the following alternative detection methods.

4b. Visualize proteins with Ponceau S stain (*UNIT B3.3*). After staining with Ponceau S, wash the membrane with water, place under clear plastic wrap, and mark bands with a soft pencil by outlining the protein band through the plastic. Completely destain the membrane with water (a permanent indentation made by the pencil will be left on the membrane).

4c. For visualizing bands after partial drying of the membrane, fill a glass dish with 50% methanol and place the dry membrane on the surface of the solution. Do not submerge the membrane. Identify protein bands (portions of the membrane containing proteins will wet more quickly and dry slower than areas that do not contain protein). Remove the membrane from the surface of the methanol solution and place on a clean glass surface. Cover the membrane with clear plastic wrap and use a pencil to mark the protein bands as described in step 4b.

5. Excise the band(s) of interest by carefully cutting the membrane with a clean razor blade or scalpel.

   Do not allow the membrane to dry during this process. Keep the membrane wet (e.g., submerged in water) at all times (unless using the partial drying procedure).

6. Place the excised membrane band in a 1.5-ml microcentrifuge tube containing 0.2 to 0.5 ml of Triton/SDS elution buffer for every square centimeter of membrane.

Microcentrifuge the sample 10 min at maximum speed, at room temperature, to release the protein from the membrane.

7. Remove supernatant, place it in a new microcentrifuge tube, and microcentrifuge again to remove any particulate material.

   Multiple extractions with the elution mixture significantly aid quantitative recovery. In most cases, three extractions remove 90% to 100% of the protein from the membrane.

ALTERNATE PROTOCOL 4

PROTEIN ELUTION FROM PVDF MEMBRANES USING ACIDIC EXTRACTION WITH ORGANIC SOLVENTS

Blotted proteins are usually of high purity and thus good candidates for additional characterization. If the protein cannot be further characterized on the blot and contamination of samples with large amounts of detergent (Basic Protocol 2) is unacceptable, acidic elution with organic solvents may be an alternative. The use of organic solvents limits this protocol to PVDF membranes, which have high chemical resistance. This protocol may produce highly variable recoveries for different proteins and is best suited for relatively low-molecular-weight proteins and peptides (<50 kDa).

Additional Materials *(also see Basic Protocol 2)*

Extraction solution (see recipe)
50% methanol
1:2 (v/v) acetonitrile/formic acid

NOTE: All solutions should be made with high-purity water and can be stored at room temperature for at least 1 month.

1. Rinse the desired number of 1.5-ml microcentrifuge tubes (two tubes per sample) three times with extraction solution, and air dry under an aluminum foil dust cover.

   Minimize possible contamination with dust at all times.

2. Visualize and mark the protein bands by partial drying of the membrane using 50% methanol (see Basic Protocol 2, step 4c). Use a scalpel or razor blade to cut out the protein bands.

3. Place pieces of membrane containing the protein of interest in a clean microcentrifuge tube.

   Combining several bands from multiple gels is usually necessary. Use a pair of tweezers cleaned with 100% methanol to transfer the membrane pieces to the microcentrifuge tube. Avoid contaminating the membrane pieces and microcentrifuge tube with airborne dust or residues from fingers.

4. Add 200 µl extraction solution to each microcentrifuge tube.

   An alternative mixture of 1:2 acetonitrile/formic acid seems to be as efficient as the extraction solution described above for some proteins.

5. Agitate tubes for 48 hr at 4°C.

6. Repeat the extraction using 1:2 acetonitrile/formic acid. Combine the extracts.

   Multiple extractions (up to three) with the same or different solvent mixtures are often beneficial. The extracts should be combined and kept on ice at all times.

7. Lyophilize protein extracts to provide highly purified samples suitable for most subsequent experiments.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Extraction solution (40% acetonitrile/1% trifluoroacetic acid)

Add 1.0 ml trifluoroacetic acid to 59 ml high-purity water, then add 40 ml acetonitrile. Store at room temperature. Solution is stable for at least 1 month in a tightly sealed container.

Transfer buffer, 20×

200 mM Tris base
2.0 M glycine
Do not adjust pH of final solution (stable at room temperature for at least 1 month)

(Prepare 1× transfer buffer by mixing 200 ml 20× stock, 400 ml methanol, and water to 4 liters.) Prepare 1× transfer buffer on the same day of use. Keep at room temperature.

Triton/SDS elution buffer

50 mM Tris·Cl, pH 9.0 (*APPENDIX 2A*)
2% (w/v) SDS (*APPENDIX 2A*)
1% (v/v) Triton X-100

Prepare solution on the same day of use. Keep at room temperature.

COMMENTARY

Background Information

The transfer of proteins from polyacrylamide gels onto blot membranes offers many benefits. Transferred proteins can be eluted from the membrane, probed with antibodies (immunoblotting), used for N-terminal protein sequencing as well as other structural analyses including amino acid analysis, protease cleavage, and chemical cleavages, or stained by a variety of highly sensitive techniques. In addition, several manipulations can be performed on the same blot by loading samples in multiple lanes on the gel before electroblotting.

Prestained protein standards are useful guides for cutting the membrane prior to different analysis procedures, such as using one part for immunoblotting and the other for protein staining. Prestained radiolabeled standards are useful for aligning the blot membrane with autoradiograms. Prestained standards will migrate differently on the gel compared with the unmodified standard proteins, usually showing higher molecular weight.

During electrotransfer, proteins migrate out of gels in an electric field according to the charge on the protein. Most electrotransfers employ a tank transfer apparatus (Fig. B3.2.2) in which the gel/membrane transfer sandwich is mounted in a cassette and placed in a tank of Tris/glycine/methanol transfer buffer. The semidry transfer system (Fig. B3.2.3) is an alternative. The major advantages of the semidry system are its use of substantially less buffer and the shortening of transfer time due to the close positioning of the electrodes, which produces a high field strength with minimal heating. A variety of transfer membranes, which can effectively bind proteins based on different types of protein-membrane interactions, are commercially available. These include PVDF, nitrocellulose, and nylon membranes as well as a number of derivatized membranes.

PVDF membranes bind proteins primarily through hydrophobic interactions and are commonly used for their chemical resistance as well as physical stability. High-affinity PVDF membranes such as Trans-Blot (Bio-Rad), ProBlott (Perkin-Elmer), and Immobilon-P^{SQ} (Millipore) are preferred for blots intended for use in N-terminal protein sequencing, whereas low-retention membranes such as Immobilon-P (Millipore) may produce lower background in both immunoblotting and common staining procedures. In addition, low-retention membranes are preferred when proteins will be extracted from the membrane.

Nitrocellulose binds proteins primarily by hydrophilic and/or electrostatic interactions and for this reason is less sensitive than PVDF membranes to the concentration of SDS in both

the gel and transfer buffer. Its tolerance to SDS allows relatively good recovery of poorly migrating proteins because higher concentrations of SDS can be left in the gel or added to the transfer buffer without adversely affecting protein binding to the membrane. In addition, nitrocellulose is often used for immunoblots, although PVDF membranes have replaced nitrocellulose for this application in many laboratories. The major limitations of nitrocellulose are its poor binding of low-molecular-weight proteins and peptides, mechanical weakness, and lack of resistance to organic solvents.

PVDF can be derivatized to modify the nature of protein-membrane interactions and to potentially increase the strength of protein binding. Derivatization most commonly involves adding a charge to the membrane so that proteins will be held on the membrane by electrostatic charges as well as the usual hydrophobic or hydrophilic interactions. The procedure for electrotransfer onto derivatized membranes is essentially identical to that for PVDF or nitrocellulose membranes described in this unit. Although derivatized membranes are better for binding RNA and DNA, most derivatized membranes have not offered clear advantages for blotting proteins.

One commercially available derivatized PVDF membrane that has potentially useful binding properties for proteins is Immobilon-CD (Millipore), which has been derivatized to produce a cationic surface so that it behaves like an ion-exchange support (Patterson et al., 1992). In addition, the membrane is treated to prevent nonspecific protein adsorption. The well-defined nature of the protein/Immobilon-CD interaction allows good recoveries of electroblotted proteins under relatively mild conditions. Proteins may be recovered from CD membranes nearly quantitatively using denaturing detergent solutions such as 4 M guanidine hydrochloride/0.1% Triton X-100. Proteins can be fragmented using a variety of enzymes and proteases either directly on the Immobilon-CD membrane or after elution. If the proteins are to be digested after elution, the enzymes should be tested for compatibility with the elution buffer.

Proteins can also be eluted from Immobilon-P membranes, and two procedures for doing so are described in this unit: protein elution using detergents (Basic Protocol 2; Szewczyk and Summers, 1988) and acidic elution with organic solvents (Alternate Protocol 4; D.W.S., unpub. observ.). Although gel electrophoresis combined with subsequent electroblotting is often the most powerful procedure for microscale protein isolation, identification, and characterization, one limitation of this system is that subsequent elution of proteins from PVDF membranes in good yields is often difficult.

Critical Parameters and Troubleshooting

The protocols listed in this unit should be sufficient for the transfer of most proteins. The efficiency of protein transfer is easily assessed by staining both the transfer membrane and the gel after transfer. When difficulty is encountered in the transfer recovery of a specific protein, several factors can be considered to improve transfer efficiency.

A first consideration is the type of gel used to separate proteins for transfer. Higher percentages of acrylamide make the transfer of proteins out of the gel more difficult. Gel thickness also affects transfer: the thicker the gel, the farther the protein has to migrate to reach the membrane. In general, effective transfer of proteins out of gels up to 1.5 mm thick can be achieved; thicker gels, although having increased protein capacity, offer little advantage owing to reduced removal of proteins from the thicker gel matrix.

In this unit, the transfer buffer described is essentially half-strength Towbin buffer (Towbin et al., 1979). This buffer contains a mixture of Tris and glycine and is the buffer of choice if the electroblotted proteins will be used for sequence analysis. Tris, glycine, and most other low-molecular-weight buffer components do not normally bind to PVDF membranes and can be completely removed by thorough rinsing with high-purity water. However, incomplete rinsing or partial drying of the membrane prior to rinsing can result in high residual levels of these compounds, which could be detrimental for sequence analysis.

The amino groups in the buffer act as scavengers for contaminants that could potentially modify proteins. Several simple precautions noted in Alternate Protocol 1 can be taken to minimize side reactions of proteins during electrophoresis and electroblotting (Speicher, 1989). If possible chemical modifications are not critical, several other buffers may be used. CAPS buffer (cyclohexylaminopropanesulfonic acid; LeGendre and Matsudaira, 1988) has a high pH (pH 11) and low ionic strength and, therefore, because of its low conductivity, permits the use of high electric fields. For the

transfer of very basic proteins that bind SDS poorly, high pH may prove advantageous in keeping the total net protein charge negative; however, the high pH of the CAPS buffer can also lead to deamination of sensitive side chains. Other types of electrotransfer buffers such as Tris/borate (Vandekerckhove et al., 1985) do not differ significantly in overall performance from the Tris/glycine buffer and may be used if N-terminal protein blockage is not a major concern.

Selection of membrane type is dependent on the ultimate use of the transferred protein, although high-retention PVDF membranes are most often chosen because they bind proteins more tightly than nitrocellulose membranes and have greater chemical and mechanical stability. For immunoblots, either nitrocellulose or Immobilon-P (Millipore) are preferred by some investigators because they produce a lighter background than high-retention PVDF membranes.

It is not uncommon in electroblot procedures to observe less than optimal transfer of proteins. If the membrane is entirely blank and little or no protein is left in the gel after transfer, it is likely that the electrodes were connected in a reversed order or that the membrane was placed on the wrong side of the gel. Blank spots on membranes are caused by air bubbles, which block the flow of current.

Another problem often encountered with poor transfer of proteins relates to the amount of SDS in the gel, which coats the proteins and improves mobility during electrotransfer. Nitrocellulose membranes are not sensitive to the amount of SDS in the gel and can even tolerate addition of SDS to the transfer buffer to encourage transfer of recalcitrant proteins. PVDF membranes are more sensitive to excess SDS, which can inhibit protein binding to the hydrophobic membrane; nevertheless, in a limited number of cases, addition of SDS to the transfer buffer may be beneficial. Low-retention PVDF membranes are particularly sensitive to excess SDS concentrations, whereas high-retention PVDF membranes are less so. Methanol is a common component of many transfer buffers because it facilitates dissociation of bound SDS from proteins. Therefore, if proteins transfer from the gel efficiently but do not bind well to PVDF membranes, the methanol concentration can be increased to 20% and/or the gel can be preequilibrated in transfer buffer for 15 to 30 min prior to transfer to reduce the SDS concentration (Mozdzanowski et al., 1992). Conversely, if proteins remain in the gel after transfer, the methanol concentration can be reduced or eliminated, and if necessary a low concentration of SDS (0.005%) can be added to the transfer buffer to facilitate electrotransfer from the gel.

If proteins have transferred efficiently but the stained membrane has blurred bands or a swirled pattern, it is likely that there was insufficient contact between the gel and membrane during transfer. The transfer sandwich should be held together firmly both to avoid having the gel shift position during transfer and to ensure close contact between the gel and membrane. Another common problem with transfers is the presence of bright white spots within protein bands on the membrane. These are due to air bubbles trapped between the gel and membrane or, in the case of PVDF membranes, possibly due to local drying of the membrane during transfer unit assembly. As indicated in the protocols, it is very important to check for air bubbles before electrotransfer. Air bubbles can be rolled out from the two surfaces by using a test tube, or the membrane can be carefully lifted to release the bubble and replaced without shifting the position of either gel or membrane.

Anticipated Results

Proteins in the middle two-thirds of the gel usually transfer to high-retention PVDF membranes with an average yield of 50% to 80%. The protein pattern on PVDF membranes stained with amido black or Coomassie blue should closely resemble the pattern on duplicate lanes of the gel stained with Coomassie blue. The staining intensity on the blot should be slightly higher than on the gel because the proteins are concentrated on the surface of the blot rather than distributed throughout the thickness of the gel. Proteins below the dye front usually will not be recovered on the membrane. Proteins in the top 20% of the gel are often incompletely transferred out of the gel.

Time Considerations

Assembling the transfer unit takes ~30 min. Electroblotting can be completed in 2 to 4 hr in most cases using a tank transfer unit and in ~1 hr using a semidry blotting unit.

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Detection of Proteins on Blot Membranes

Staining of blot transfer membranes permits visualization of proteins and allows the extent of transfer to be monitored. In the protocols described in this unit, proteins are stained after electroblotting from one-dimensional or two-dimensional polyacrylamide gels to blot membranes such as polyvinylidene difluoride (PVDF), nitrocellulose, or nylon membranes (*UNIT B3.2*). PVDF is the preferred, more universal membrane and is emphasized here; however, most stains work similarly on nitrocellulose, and many can be used on alternative blotting membranes.

The first six Basic Protocols describe the use of six general protein stains—amido black, Coomassie blue, Ponceau S, colloidal gold, colloidal silver, and India ink. In addition, the fluorescent stains fluorescamine and IAEDANS, which covalently react with bound proteins, are described in Basic Protocol 7 and the Alternate Protocol. Table B3.3.1 lists approximate detection limits for each nonfluorescent stain as well as membrane compatibilities. The dimensional stability of blotted membranes facilitates direct, precise comparisons of staining patterns using different detection methods. For example, protein staining can be directly compared with immunoreactivity by staining one portion of a blot with a general protein stain while subjecting another portion containing duplicate samples to immunoblotting. When it is desirable to cut replicate lanes from the blot prior to any staining, prestained standards provide very useful visual reference points.

NOTE: High-purity water (from a Milli-Q purification system or equivalent) should be used throughout the protocols, and all plastic and glass boxes must be thoroughly cleaned before use to avoid staining artifacts. Membranes should be handled only by the edges with forceps. All steps should be performed at room temperature (unless otherwise described) and with gentle agitation. Use of an orbital shaker is recommended for steps that take longer than 1 min. If a PVDF membrane is allowed to dry after transfer, wet for 5 sec in 100% methanol and rinse with water before staining. Volumes of stain, destain, and wash solutions should be sufficient to cover the membrane and allow it to float freely. Unless noted otherwise, solutions may be stored for several months at room temperature.

Table B3.3.1 Staining Sensitivities and Membrane Compatibilities for Nonfluorescent Stains[a]

Stain	Minimum amount detected[b]	Membrane type[c]		
		PVDF	Nitrocellulose	Nylon
Amido black	50 ng	+	+	+
Coomassie blue	50 ng	+	−	+
Ponceau S	200 ng	+	+	+
Colloidal gold	2 ng	+	+	−
Colloidal silver	5 ng	+	+	−
India ink	5 ng	+	+	−

[a]Sensitivity of fluorescent stains is very dependent on protein amino acid composition.

[b]Minimum amount detected based on amount of protein loaded onto gel. The actual amount on the blot will be slightly lower because of losses during electrotransfer. Values are based on use of a full-sized gel (11 cm × 16 cm × 1.5 mm). Sensitivity will be ~2 to 5 times higher when minigels (8 cm × 10 cm × 1.0 mm) are used because the protein bands are concentrated on a smaller area of membrane.

[c]+ indicates stains well; − indicates membrane not compatible.

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BASIC PROTOCOL 1

AMIDO BLACK STAINING

Amido black is used to stain proteins on blot transfer membranes. Transferred proteins (>50 ng/band) appear as dark blue bands on a light blue background. Amido black has a sensitivity similar to that of Coomassie blue, but it stains faster. It is the preferred stain for protein sequencing and in situ cleavage of proteins for determining internal sequences because the mild staining and destaining conditions minimize the likelihood that any protein will be extracted during the procedures.

Materials

Protein sample electroblotted to PVDF, nitrocellulose, or nylon membrane (*UNIT B3.2*)
Amido black 10B stain: 0.1% (w/v) amido black (naphthol blue black 10B, Sigma) in 10% (v/v) acetic acid
5% (v/v) acetic acid
Plastic boxes

1. Place blot transfer membrane in a plastic box and wash with water three times for 5 min each.
2. Stain membrane with amido black 10B stain for 1 min.

   Longer staining times only increase background and hence decrease sensitivity.

3. Destain the membrane with 5% acetic acid twice for 1 min each.
4. Rinse the membrane with water twice for 10 min each, then air dry.

BASIC PROTOCOL 2

COOMASSIE BLUE R-250 STAINING

Coomassie blue R-250 can be used with most types of blot membranes except nitrocellulose (high concentrations of organic solvents can dissolve nitrocellulose membranes). Coomassie blue has a similar sensitivity to amido black. Coomassie blue–stained proteins (>50 ng/band) appear as dark blue bands against a light blue background. The sequence of washing with water, staining, and destaining is similar to that for amido black staining (Basic Protocol 1), but the staining step is lengthened to 5 min.

Materials

Blot transfer membrane (*UNIT B3.2*)
Coomassie blue stain: 0.025% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in 40% methanol/7% acetic acid (v/v)
50% methanol/7% acetic acid (v/v)
Plastic box

1. Place blot transfer membrane in a clear plastic box. Wash with water three times for 5 min each.
2. Stain membrane with Coomassie blue stain for 5 min.

   If proteins on PVDF membranes are to be subjected to N-terminal sequencing, acetic acid should be omitted from the staining and destaining solutions to minimize protein extraction.

3. Destain membrane with 50% methanol/7% acetic acid for 5 to 10 min.
4. Rinse with water several times, then air dry.

PONCEAU S STAINING

BASIC PROTOCOL 3

Ponceau S is the least sensitive general protein stain described here. Transferred proteins (>200 ng/band) appear as red bands on a pink background. Major advantages of Ponceau S staining are that it is simple, rapid, and reversible. If desired, essentially all of the stain can be removed by extended destaining as described in steps 4 to 6. This can be particularly advantageous if the blot is to be reused after initial protein staining for a second detection method such as immunoblotting.

Materials

Blot transfer membrane (*UNIT B3.2*)
Ponceau S stain: 0.5% (w/v) Ponceau S (Sigma) in 1% (v/v) acetic acid
200 μM NaOH/20% (v/v) acetonitrile
Plastic box

1. Place blot transfer membrane in a plastic box and wash with water three times for 5 min each.
2. Stain membrane with Ponceau S stain for 30 sec to 1 min.
3. Destain membrane with several changes of water for 30 sec to 1 min each, then air dry. If the stain is to be extracted from protein bands (steps 4 to 6) prior to employing a second detection method, omit drying.

   Do not overdestain because the protein bands will be difficult to detect. Stop destaining when the background has a very slight pink tinge.

4. Make a permanent record of the staining pattern by photocopying or photographing the blot. Alternatively, mark the positions of the bands of interest directly on the blot by overlaying the wet blot with plastic wrap or a plastic bag and using a pencil or pen to outline the bands. Press with moderate pressure to make a permanent indentation on the membrane.
5. Extract Ponceau S stain from the protein bands with 200 μM NaOH/20% acetonitrile for 1 min.
6. Wash membrane with water three times for 5 min each, then air day.

COLLOIDAL GOLD STAINING

BASIC PROTOCOL 4

Colloidal gold is a highly sensitive stain. AuroDye (Amersham) is a ready-to-use commercial source of colloidal gold stain in a low-pH buffer. AuroDye should be used without dilution. Transferred proteins (>2 ng/band) will appear as red bands on a pink background. A higher signal may be obtained with alkali treatment of the membrane prior to staining by washing the membrane with 1% KOH followed by several rinses with phosphate-buffered saline (PBS). Glass rather than plastic boxes must be used to hold the membranes and solutions. Glass is easier to clean than plastic and is less likely to give artifacts. Ideally, a set of glass trays should be dedicated to the procedure.

Materials

Blot transfer membrane (*UNIT B3.2*)
Tween 20 solution: 0.3% (v/v) Tween 20 in PBS (prepare solution fresh weekly and store at 4°C)
AuroDye colloidal gold reagent (Amersham; store at 4°C)
Glass box

1. Place blot transfer membrane in a glass box. Wash with water three times for 5 min each.
2. Incubate membrane with Tween 20 solution for 30 min at 37°C with gentle agitation.
3. Wash membrane with Tween 20 solution three times for 5 min each at room temperature.
4. Rinse membrane several times with water.
5. Stain membrane with AuroDye for 2 to 6 hr (until desired color formation).

   Use only enough stain to cover the membrane completely.

6. Rinse membrane thoroughly with water, then air dry.

BASIC PROTOCOL 5

COLLOIDAL SILVER STAINING

Colloidal silver is a more economical stain than colloidal gold. The staining procedure is rapid, although the ferrous sulfate solution must be prepared immediately before use. Transferred proteins (>5 ng/band) appear as black bands on a light brown background for nitrocellulose and on a dark background for PVDF.

Materials

Blot transfer membrane (*UNIT B3.2*)
40% (w/v) sodium citrate (store at 4°C for several months)
20% (w/v) ferrous sulfate ($FeSO_4 \cdot 7H_2O$), prepared fresh
20% (w/v) silver nitrate (store at 4°C for several months)
Glass box

1. Place blot transfer membrane in a glass box and wash with water three times for 5 min each.
2. Add 5 ml of 40% sodium citrate and 4 ml of 20% ferrous sulfate to 90 ml water. Stir vigorously and slowly add 1 ml of 20% silver nitrate over ~1 to 2 min to form a suspension.
3. Immediately use the suspension to stain the membrane for ~5 min.

   The staining suspension should be used within 30 min.

4. Rinse membrane with water, then air dry.

BASIC PROTOCOL 6

INDIA INK STAINING

India ink is used to stain electroblotted proteins on blot transfer membranes. Transferred proteins (>5 ng/band) appear as black bands on a gray background. Sensitivity may be enhanced by brief alkali treatment of the membrane with 1% KOH followed by several rinses with PBS.

Materials

Blot transfer membrane (*UNIT B3.2*)
Tween 20 solution: 0.3% (v/v) Tween 20 in PBS (prepare solution fresh weekly and store at 4°C)
India ink solution: 0.1% (v/v) India ink (Pelikan 17 black) in Tween 20 solution (store 1 month at room temperature)
Plastic box

1. Place blot transfer membrane in a plastic box. Wash with water three times for 5 min each.

2. Wash membrane with Tween 20 solution four times for 10 min each.
3. Stain membrane with India ink solution for 2 hr or overnight.
4. Rinse with water until an acceptable background is obtained, then air dry.

FLUORESCAMINE LABELING

BASIC PROTOCOL 7

Fluorescamine, or 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione, is used to introduce a fluorescent label on electroblotted proteins via reaction with free amines. Transferred proteins are visualized on blot transfer membranes with UV light. This stain can be very sensitive and can be used in conjunction with a second detection method such as immunoblotting (also see Basic Protocol 3). However, the protein is irreversibly modified because fluorescamine reacts with available amino groups (i.e., lysines and the protein N terminus if it was not previously blocked).

Materials

Blot transfer membrane (*UNIT B3.2*)
Sodium bicarbonate solution: 100 mM sodium bicarbonate in 0.3% (v/v) Tween 20, pH 9.0 (prepare fresh weekly and store at 4°C)
Fluorescamine stain: 0.25 mg/ml fluorescamine (Sigma) in sodium bicarbonate solution (prepare fresh daily)
Plastic box

1. Place blot transfer membrane in a plastic box. Wash with water three times for 5 min each.
2. Wash membrane with sodium bicarbonate solution twice for 10 min each.
3. Label protein bands with fluorescamine stain for 15 min. Use enough staining solution to cover the membrane completely.
4. Wash membrane with bicarbonate solution three times for 5 min each.
5. Rinse membrane several times with water.
6. Visualize transferred proteins with UV light.

IAEDANS LABELING

ALTERNATE PROTOCOL

N-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS or 1,5-I-AEDANS) is used for fluorescent labeling of electroblotted proteins on blot transfer membranes. Because IAEDANS reacts with free cysteines, disulfides in the sample must first be reduced with dithiothreitol (DTT). Transferred protein bands are visualized under UV light.

Additional Materials *(also see Basic Protocol 7)*

DTT solution: 200 mM dithiothreitol (DTT) in 100 mM Tris·Cl, pH 8.6 (*APPENDIX 2A*; prepare immediately before use)
100 mM Tris·Cl, pH 8.6 (*APPENDIX 2A*)
N-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS; Sigma; store desiccated in the dark at −20°C)
Glass box

1. Place blot transfer membrane in a glass box. Wash with water three times for 5 min each.
2. Incubate membrane with DTT solution for 30 min to reduce disulfides in the sample.
3. Wash membrane with 100 mM Tris·Cl (pH 8.6) three times for 5 min each.

This washing step probably could be deleted if proteins have been electroblotted from a reducing gel (i.e., containing DTT and/or 2-mercaptoethanol). However, it is advisable to include this simple step routinely as some oxidation may occur during electrotransfer or on the blotted membrane itself during drying or storage.

4. Dissolve 86 mg IAEDANS (2 mM final concentration) in 100 ml of 100 mM Tris·Cl (pH 8.6).

   This step should be carried out in the dark (solution should be made fresh daily).

5. Add IAEDANS solution to the membrane and allow the reaction to proceed in the dark for 30 min with shaking.
6. Wash membrane with 100 mM Tris·Cl (pH 8.6) twice for 5 min each. Thoroughly rinse membrane with water to remove excess reagent.
7. Visualize the transferred proteins under UV light.

COMMENTARY

Background Information

The recent rapid expansion in the use of electrophoretic transfer of separated proteins to different types of membranes has necessitated the adaptation of existing protein staining techniques to transfer membranes. On-blot staining techniques serve multiple purposes, including detection of proteins for structural analysis and use in parallel with antibody reactivity to correlate precisely immunoreactivity with protein staining patterns. For the latter purpose an advantage of on-blot staining is that duplicate sections of membrane can be cut out and one section used for immunoblotting (*UNIT B3.4*) while a second section with duplicate lanes is stained with a general protein stain. The two pieces can then be precisely realigned. In contrast, a direct comparison of an immunoblot with a stained polyacrylamide gel is much less precise because of shrinking and swelling of the gel.

The most common membranes used for electroblotting are polyvinylidene difluoride (PVDF) and nitrocellulose. PVDF membranes have become increasingly popular because they are easy to handle and store, whereas nitrocellulose membranes are brittle and tend to break easily when dry. A number of PVDF membranes are now commercially available that have subtle but important differences in protein binding properties resulting from different proprietary manufacturing processes. For example, Bio-Rad Trans-Blot or Millipore Immobilon-P^{SQ} PVDF membranes generally show higher protein binding capacities and higher binding affinities compared to Immobilon-P (Mozdzanowski and Speicher, 1992). Use of high-retention PVDF membranes usually results in higher and more consistent electroblotting recoveries of most proteins than the use of either low-retention PVDF membranes or nitrocellulose. However, high-retention PVDF membranes tend to exhibit higher staining backgrounds, and it is more difficult to extract proteins or peptides from such membranes.

The protocols for staining with amido black, Coomassie blue, Ponceau S, and AuroDye follow the suppliers' recommendations. It should be noted that when staining PVDF membranes with Coomassie blue before N-terminal sequencing, omitting acetic acid from both the stain and destain solution is recommended to minimize potential extraction of protein from the membrane (Speicher, 1989).

The protocol for India ink staining of electroblotted proteins is essentially that of Hancock and Tsang (1983). Different brands of India ink may be used, but staining sensitivity may vary as a result.

The colloidal gold stain is the most sensitive membrane stain described here. As an alternative to commercially available colloidal gold stains, Moeremans et al. (1985) describe methods for preparing gold and iron solutions.

Fluorescent labels are advantageous because they can be used not only for sequential detection methods on the same blot with minimal potential interference, but also for detection prior to protein extraction from the membrane. For example, after visualization of proteins with a fluorescent label, the blot can be photographed and specific bands marked with a pencil, either directly on the membrane or through a plastic bag. The latter method leaves a permanent indentation on the membrane. The blot can then be probed with antisera (i.e., immunoblotted; *UNIT B3.4*).

The protocol for fluorescamine labeling is based on the procedure described by Vera and Rivas (1988). Fluorescamine itself is not strongly fluorescent; however, when combined with the primary amines of proteins (i.e., N termini and lysine residues) it yields a highly fluorescent product. IAEDANS is an iodoacetic acid analog containing a naphthalene ring and is fluorescent under UV light. When the sample protein is reduced either prior to gel electrophoresis or with DTT after blotting, all cysteines are potentially available for reaction with IAEDANS. When the protein is not reduced, some cysteines may be involved in disulfide bonds and therefore not available for reaction. Additionally, some cysteines may be sterically inaccessible because of adsorption to the membrane and therefore will not react.

An additional visualization technique for PVDF membranes is transillumination, described by Reig and Klein (1988). In that technique, the membrane is dried at room temperature, then wet with 20% methanol and viewed on a white light box. Protein bands appear as clear areas. Sensitivity is usually comparable to that of Coomassie blue staining.

Critical Parameters and Troubleshooting

High-quality water (from a Milli-Q purification system or equivalent) should be used throughout these protocols. All plastic and glass boxes must be thoroughly cleaned by rinsing with water before use to avoid staining artifacts. Blot membranes should be handled by the edges only with gloves or, preferably, with forceps. This precaution is most critical for the more sensitive stains (i.e., colloidal gold, colloidal silver, and India ink). When using PVDF membranes, it is especially critical that the membrane does not dry between steps. If drying occurs, wet the PVDF membrane for 5 sec with 100% methanol, then rinse several times with water.

A brief alkali treatment can enhance staining with India ink or colloidal gold. In a procedure described by Sutherland and Skerritt (1986), the membrane is washed with 1% (w/v) KOH for 5 min followed by several rinses with PBS. The alkali treatment can easily be incorporated at the beginning of the procedures if desired.

Anticipated Results

Approximate detection limits and membrane compatibilities are listed in Table B3.3.1. It should be noted that detection limits may vary with gel size and the percentage of polyacrylamide in the gel used in preparation of the blot transfer membrane. The sensitivity of fluorescent stains is related to the number of reactive amino groups (fluorescamine) or cysteine residues (IAEDANS) present in the protein of interest.

Time Considerations

The total time required for staining with amido black, Coomassie blue, and Ponceau S is 30 min to 1 hr; colloidal gold requires 4 to 6 hr; colloidal silver requires 1 hr; whereas staining with India ink requires 2 hr to overnight. Fluorescent labeling requires ~1 hr. The optional alkali enhancement (see Critical Parameters and Troubleshooting) requires an additional 30 min at the beginning of the India ink and colloidal gold staining procedures.

Literature Cited

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Speicher, D.W. 1989. Microsequencing with PVDF membranes: Efficient electroblotting, direct protein adsorption and sequencer program modifications. *In* Techniques in Protein Chemistry (T.E. Hugli, ed.) pp. 24-35. Academic Press, San Diego.

Sutherland, M.W. and Skerritt, J.H. 1986. Alkali enhancement of protein staining on nitrocellulose. *Electrophoresis* 7:401-406.

Vera, J.C. and Rivas, C. 1988. Fluorescent labeling of nitrocellulose-bound proteins at the nanogram level without changes in immunoreactivity. *Anal. Biochem.* 173:399-404.

Key References

Moeremans et al., 1985. See above.

Describes a method for preparation of colloidal metal stains.

Vera and Rivas, 1988. See above.

Describes the use of multiple detection methods.

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Immunoblot Detection

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and in selected cases with reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME); some antibody epitopes are destroyed if reducing conditions are used. Following solubilization, the material is separated by SDS-PAGE (using either one- or two-dimensional gels; *UNIT B3.1*). The antigens are then electrophoretically transferred in a tank or a semidry electroblotting unit to a nitrocellulose, polyvinylidene difluoride (PVDF), or nylon membrane (*UNIT B3.2*). When nitrocellulose or PVDF membranes are used, the process can be monitored by a reversible staining procedure with Ponceau S (*UNIT B3.3*). After staining, protein bands on the membrane can be photographed and/or the positions of the detected proteins can be marked with indelible ink (e.g., Paper-Mate pen). The membrane is then completely destained by soaking in water for an additional 10 min.

At this point the transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After being probed with primary antibody, the membrane is washed and the antibody-antigen complexes are identified using horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes coupled to the secondary anti-immunoglobulin-G (anti-IgG) antibody (e.g., goat anti-rabbit IgG). The enzymes are attached directly (Basic Protocol) or via an avidin-biotin bridge (Alternate Protocol) to the secondary antibody. Chromogenic or luminescent substrates (Support Protocols 1 and 2) are then used to visualize the activity.

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

BASIC PROTOCOL

After electrophoretic transfer to the membrane (*UNIT B3.2*), the immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is first immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, the membrane is placed in a solution containing an antibody directed against the antigen (primary antibody). The blot is washed and then exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Support Protocols 1 and 2) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is a common alternative to protein blocking agents for use with nitrocellulose or PVDF filters.

Materials

Membrane with transferred proteins (*UNIT B3.2*)
Blocking buffer (see recipe) appropriate for membrane and detection protocol
Primary antibody specific for protein of interest
TTBS (nitrocellulose or PVDF) *or* TBS (neutral or positively charged nylon; see recipes for both solutions)
Secondary antibody conjugate: horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-anti-Ig (Cappel, Vector, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25-µl aliquots until use)

Contributed by Sean Gallagher
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Heat-sealable plastic bags
Powder-free gloves
Plastic box

Additional reagents and equipment for chromogenic or luminescent visualization (see Support Protocol 1 or see Support Protocol 2)

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

   Usually 5 ml buffer is sufficient for two to three membranes (14 × 14–cm size).

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. B3.4.1; Cooper and Paterson, 1995; Andrew and Titus, 1991a,b,c, 1993), 1/10 to 1/100 for hybridoma supernatants (Yokoyama, 1991a), and ≥1/1000 for murine ascites fluid containing monoclonal antibodies (Yokoyama, 1991b). Ten- to one-hundred-fold higher dilutions can be used with alkaline phosphatase– or luminescence–based detection systems. Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary, depending on conjugate used.

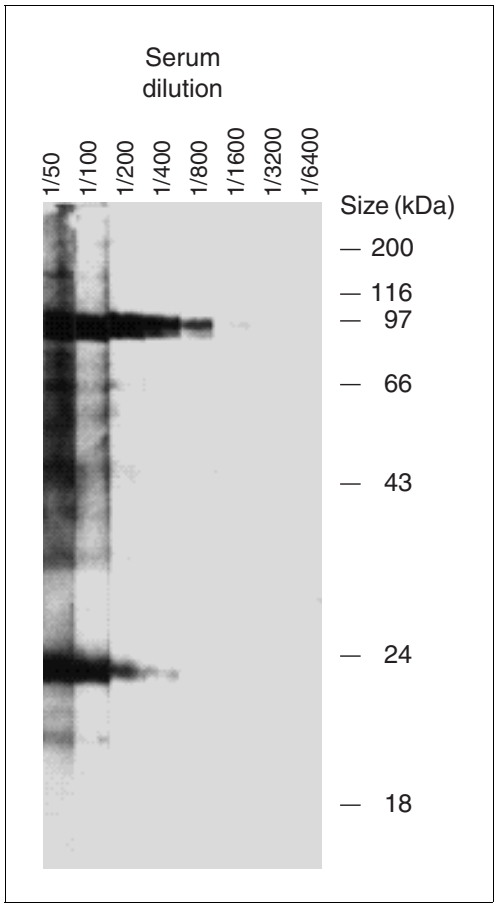

Figure B3.4.1 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. The blot was developed with HRP-coupled avidin-biotin reagents according to the Alternate Protocol and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash 4 times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.
5. Dilute secondary antibody HRP- or AP-anti-Ig conjugate in blocking buffer.

   Commercially available enzyme-conjugated secondary antibody is usually diluted 1/200 to 1/2000 (i.e., 20 µl/ml to 2 µl/ml) prior to use (Harlow and Lane, 1988).
6. Place membrane in fresh heat-sealable plastic bag, add diluted HRP- or AP-anti-Ig conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

   When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.
7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Support Protocol 1 or see Support Protocol 2).

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

ALTERNATE PROTOCOL

The following procedure is based on the Vectastain ABC kit from Vector (*SUPPLIERS APPENDIX*). It uses an avidin-biotin complex to attach horseradish peroxidase (HRP) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity because multiple reporter enzymes are bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein blocking agents when using nitrocellulose or PVDF membranes.

Additional Materials *(also see Basic Protocol)*

Vectastain ABC (HRP) *or* ABC-AP (AP) kit (Vector) containing the following: reagent A (avidin), reagent B (biotinylated HRP or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥2 hr at 37°C.

   TTBS is well suited for avidin-biotin systems. For nylon, protein binding agents are recommended. Because nonfat dry milk contains residual biotin that will interfere with the immunoassay, its use must be restricted to the blocking step only.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.
2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

   Dilutions of sera containing primary antibody generally range from 1/100 to 1/10,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate concentration of primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., with a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher & Schuell or Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. B3.4.1).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Reseal bag and incubate 30 min at room temperature with gentle rocking.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane 3 times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting 2 drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

   This dilution gives both high sensitivity and enough volume to easily cover a large (14 × 14–cm) membrane.

6. Transfer membrane to fresh plastic bag containing secondary antibody solution. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

   When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRP or -AP complex. Mix 2 drops Vectastain reagent A and 2 drops reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

   Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity. Azide is a peroxidase inhibitor and should not be used as a preservative for long-term storage of the antibody solution. Casein, nonfat dry milk, serum, and some grades of bovine serum albumin (BSA) may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; see also instructions from Vector).

8. Transfer membrane to fresh plastic bag containing avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.

   Hybridization in a plastic bag requires 5 to 10 ml avidin-biotin-enzyme solution. Membrane strips require 5 to 10 ml/strip, whereas blots from standard-sized gels (i.e., 14 × 16 cm) require 50 ml for convenient handling in a tray.

9. Develop membrane according to the appropriate visualization protocol (see Support Protocol 1 or see Support Protocol 2).

SUPPORT PROTOCOL 1

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

After incubation with primary and secondary antibody conjugates (see Basic Protocol or see Alternate Protocol), bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with horseradish peroxidase (HRP)–based immunodetection procedures, whereas BCIP/NBT is recommended for alkaline phosphatase (AP)–based procedures (see Table B3.4.1). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins and probed with antibody-enzyme complex (see Basic Protocol or see Alternate Protocol)
TBS (see recipe)
Chromogenic visualization solution (Table B3.4.1; see recipes)
Additional reagents and equipment for gel photography

1. If final membrane wash (see Basic Protocol, step 7, or see Alternate Protocol, step 9) was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

   The Tween 20 in the TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution. Bands should appear in 10 to 30 min.
3. Terminate reaction by washing membrane in distilled water. Air dry and photograph for a permanent record.

Table B3.4.1 Chromogenic and Luminescent Visualization Systems[a]

System	Reagent[b]	Reaction/detection	Comments[c]
Chromogenic			
HRP-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly on exposure to light
	DAB/$NiCl_2$[d]	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane is easily scanned
	TMB[e]	Forms dark purple stain	More stable and less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[e]; can be used with all membrane types
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRP-based	Luminol/H_2O_2/ *p*-iodophenol	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction is detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane phosphates (e.g., AMPPD, CSPD, Lumigen-PPD, Lumi-Phos 530[f])	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting)

[a]Abbreviations: AMPPD or Lumigen-PPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}]-decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.

[b]Recipes and suppliers for all reagents except TMB are listed in Reagents and Solutions. Kits containing TMB are available from Kirkegaard & Perry, TSI Center for Diagnostic Products, and Vector.

[c]See Commentary for further details.

[d]DAB/$NiCl_2$ can be used without the nickel enhancement, but sensitivity is greatly reduced.

[e]First treating nitrocellulose filters with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0) causes TMB to precipitate onto the membrane with a sensitivity much greater than that seen for 4CN or DAB and equal to or better than that for BCIP/NBT (McKimm-Breschkin, 1990).

[f]Lumi-Phos 530 contains dioxetane, $MgCl_2$, cetyltrimethylammonium bromide (CTAB), and fluorescent enhancer in a pH 9.6 buffer.

SUPPORT PROTOCOL 2

VISUALIZATION WITH LUMINESCENT SUBSTRATES

After incubation with primary and secondary antibody conjugates (see Basic Protocol and see Alternate Protocol), antigens can also be visualized with luminescent substrates. Detection with light offers both greater speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRP) systems, or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap and placed firmly against film. Exposures range from a few seconds to several hours, although typically strong signals appear within a few seconds or minutes.

Additional Materials *(also see Support Protocol 1)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*; HRP), *or* dioxetane phosphate substrate buffer (see recipe; AP)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Applied Biosystems) in dioxetane phosphate substrate buffer (see recipe), prepared just before use
Luminescent visualization solution (Table B3.4.1; see recipes)
Clear plastic wrap

NOTE: See Troubleshooting section for suggestions concerning optimization of the protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with substrate buffer each.

   For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.

2. *For AP reactions using nitrocellulose or PVDF membranes:* Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer.

   For blots of whole gels, use 50 ml Nitro-Block solution and substrate buffer; for strips, use 5 to 10 ml/strip.

   Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen-PPD concentrate. It is required for nitrocellulose and recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRP–based reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to luminescent visualization solution. Soak 30 sec (HRP reactions) to 5 min (AP reactions).

   Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step 5.

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane and seal with tape to form a liquid-tight enclosure.

   To ensure an optimal image, only one layer of plastic should be present between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

5. In a darkroom, place membrane face down onto film.

   Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that ensures a tight fit.

6. Expose film for a few seconds to several hours.

   Typically, immunoblots produce very strong signals within a few seconds or minutes; however, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr and, if needed, overnight (see Troubleshooting).

7. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Support Protocol 1).

It is possible to develop the same membrane with chromogenic substrates after luminescent visualization.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

Blocking buffer

Colorimetric detection

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; see recipe).

For neutral and positively charged nylon: Tris-buffered saline (TBS; see recipe) containing 10% (w/v) nonfat dry milk.

TTBS can be stored up to 1 week at 4°C. Prepare blocking buffer containing nonfat dry milk immediately prior to use, as the milk blocking solution is not stable.

Luminescent detection

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): 0.2% (w/v) casein (e.g., Hammarsten grade or I-Block; Applied Biosystems) in TTBS (see recipe).

For positively charged nylon: 6% (w/v) casein/1% (v/v) polyvinylpyrrolidone (PVP) in TTBS (see recipe).

For each solution: With constant mixing, add casein and PVP to warm (65°C) TTBS. Stir for 5 min, then cool. Prepare each solution just before use.

Chromogenic visualization solutions

BCIP/NBT visualization solution: Mix 33 µl NBT stock [100 mg NBT in 2 ml 70% (v/v) dimethylformamide (DMF), stored <1 year at 4°C] and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 µl BCIP stock (100 mg BCIP in 2 ml 100% DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1988). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, and Vector (SUPPLIERS APPENDIX).

4CN visualization solution: Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN). Separately mix 60 µl of 30% (w/v) H_2O_2 with 100 ml TBS (see recipe) at room temperature. Rapidly mix the two solutions and use immediately.

DAB/$NiCl_2$ visualization solution:
5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 µl DAB stock (40 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
15 µl 3% (w/v) H_2O_2
Mix just before use

CAUTION: *Handle DAB carefully, wearing gloves and mask; it is a carcinogen.*

Suppliers of chromogenic HRP substrates (4CN and DAB/$NiCl_2$) are Sigma, Kirkegaard & Perry, Moss, and Vector (SUPPLIERS APPENDIX). For selection of appropriate chromogenic solutions, and for definition of abbreviations, see Table B3.4.1.

Dioxetane phosphate substrate buffer

1 mM $MgCl_2$
0.1 M diethanolamine
0.02% (w/v) sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Traditionally, the AMPPD substrate buffer has been a solution containing 1 mM $MgCl_2$ and 50 mM sodium carbonate/bicarbonate, pH 9.6 (Gillespie and Hudspeth, 1991). The use of diethanolamine results in better light output (Western Light instructions; Applied Biosystems).

Alternatively, 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/5 mM $MgCl_2$ can be used (Sandhu et al., 1991).

Luminescent visualization solutions

Dioxetane phosphate visualization solution: Prepare 0.1 mg/ml AMPPD or CSPD (Applied Biosystems) *or* 0.1 mg/ml Lumigen-PPD (Lumigen) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use. Lumi-Phos 530 (Boehringer Mannheim or Lumigen) is a ready-to-use solution and can be applied directly to the membrane.

This concentration (240 μM) of AMPPD substrate is the minimum recommended by Applied Biosystems. Ten-fold lower concentrations can be used but require longer exposures.

Luminol visualization solution:
0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml dimethyl sulfoxide (DMSO)]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
25 μl 3% (w/v) H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). Premixed luminol substrate mix (Mast Immunosystems, Amersham, Du Pont NEN Renaissance, or Kirkegaard & Perry Lumi-GLO) may also be used. For selection of appropriate luminescent solutions, and for definition of abbreviations, see Table B3.4.1.

p-Iodophenol is an optional enhancing agent that increases light output. Luminol and p-iodophenol stocks can be stored ≤6 months at −20°C.

Tris-buffered saline (TBS)

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.9% (w/v) NaCl
Store up to several months at 4°C

Tween 20/TBS (TTBS)

0.1% (v/v) Tween 20 in Tris-buffered saline (TBS; see recipe)
Store up to several months at 4°C

COMMENTARY

Background Information

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal. However, there are several problems inherent to immunoprecipitation, including the need to radiolabel the antigen, coprecipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the

detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE or urea-PAGE (*UNIT B3.1*), followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon membranes (*UNIT B3.2*). This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies, and is highly sensitive (1 ng of antigen can be detected).

Immunoblotted proteins can be detected by chromogenic or luminescent assays (see Table B3.4.1 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRP and AP systems without the need for radioisotopes. Substrates for the latter have only recently been applied to protein blotting (see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely exceed 1 hr. Depending on the system, the luminescence can last up to 3 days, permitting multiple exposures of the same blot. Furthermore, the signal is detected by film, and varying the exposure can result in more or less sensitivity. Luminescent blots can be easily erased and reprobed because the reaction products are soluble and do not deposit on the membrane. Compared to chromogenic development, a luminescent image recorded on film is easier to photograph and to quantitate by densitometry.

AP–based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted (see Reagents and Solutions). The procedure described in Support Protocol 2 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and dilutions of primary antibody and conjugate should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1988). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution, TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink.

Two types of nylon membranes are used for transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to have a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes are popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% (w/v) nonfat dry milk in TBS is recommended for chromogenic development. During development of luminescence, however, background is a more significant problem. When compared to dry milk, purified casein has minimal endogenous AP activity (such activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP-40). Because nonfat dry milk and casein may contain biotin, which will interfere with avidin-biotin reactions, subsequent steps are done without protein blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For

example, zwitterionic detergents have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can sometimes preserve antigenicity.

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune sera or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. B3.4.1).

Because of the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow weaker signals nearby on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad, diffuse image on the film. The signal can also saturate the film, exposing it to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the AP substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Applied Biosystems Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% (w/v) casein, 1% (v/v) polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN_3, 10 mM EDTA, and phosphate-buffered saline (PBS), pH 6.8. Prior to use, the casein must be heated to 65°C to reduce AP activity in the casein itself. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If one is simultaneously testing multiple antibodies directed against a complex protein mixture, multiple bands will be visualized.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on the transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr (for high-power transfer) to overnight. Blocking, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence).

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Key References

Gillespie and Hudspeth, 1991. See above.

Describes alkaline phosphatase–based luminescent detection methods.

Harlow and Lane, 1988. See above.

Details alternative detection methods.

Schneppenheim et al., 1991. See above.

Details horseradish peroxidase–based luminescent detection methods.

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Determining the CD Spectrum of a Protein

Many of the complex phenomena associated with food processing, including cooking, involve the interactions of biopolymers. These interactions are frequently triggered by changes in the three-dimensional conformation of the biopolymers, which may be caused by changes in temperature, pH, or ionic strength, or by modifications made by hydrolytic enzymes. The understanding of such processes, necessary for their control, requires knowledge of the dependence of the conformational changes and interactions on environmental conditions. Circular dichroism (CD) can provide a sensitive indicator of these changes at the molecular level (Li-Chan, 1998), provided the sample is in the form of an optically clear solution and, for most applications, contains purified components. This unit reflects the fact that CD has been used mainly to study proteins, but it can, in principle, be used for any molecules containing a chromophore that absorbs radiation in an accessible region of the spectrum, such as nucleic acids (Gray et al., 2002), carbohydrates (Johnson, 1987), or porphyrins (Huang et al., 2000).

There are two requirements for a molecule or group of atoms in a molecule to exhibit a circular dichroism (CD) spectrum. The first is the presence of a **chromophore**—i.e., a group that can absorb radiation by virtue of the electronic configuration of its resting or ground state at room temperature. The energy absorbed results in a transition to a higher-energy or excited state, which has a different distribution of electrons around the nucleus. It can therefore interact with its environment in a way that differs from the ground state. In proteins, tryptophan, tyrosine, and phenylalanine are the main chromophores in the near-UV (240- to 320-nm) region; the peptide bond is the main chromophore in the far-UV (180- to 240-nm) region. Disulfide bonds and histidine residues are two other chromophores whose contribution to CD are, in general, less marked. Most chromophores exhibit more than one transition (e.g., see Fig. B3.5.1, Fig. B3.5.2, and Table B3.5.2) and their spectra are a composite of several absorption bands. It is an important feature of CD that, unlike optical rotation, the wavelength region within which spectra are observed is limited strictly to the wavelength region of the individual absorption bands. This confers greater specificity on spectra and allows a certain degree of assignment to particular chromophores.

The second requirement for CD is that the chromophore be in, or be closely associated with, an **optically asymmetric environment**. The chromophores in proteins are themselves not chiral and exhibit no optical activity. The phenolic group of tyrosine, for example, exhibits a CD spectrum only because it is connected to an optically asymmetric carbon atom. However, when the same group is packed in a folded protein in an environment that is asymmetric with respect to polarity, or is interacting through the phenolic hydroxyl, it exhibits a different CD spectrum that is specific to the particular environmental influences acting on the chromophore. In practice, the latter spectrum is usually much more intense than that for free tyrosine and may also differ in shape. When the peptide-bond chromophore is part of a regularly folded structure, with particular backbone angles and hydrogen-bond interactions as in an α-helix or β-sheet, it becomes closely associated with a conformationally asymmetric structure—i.e., one that cannot be superimposed on its mirror image. A CD spectrum for the protein will be generated that is a composite of the individual spectra corresponding to each of the peptide-bond absorption transitions (Fig. B3.5.1). The intensity of the spectrum may be further affected by interaction between neighboring chromophores (Bayley, 1980).

Plane-polarized radiation comprises two circularly polarized vectors of equal intensity, one right-handed and the other left-handed (Fig. B3.5.3A), which are separately measured in the CD spectrometer by means of a photoelastic modulator. A chromophore situated

Contributed by Roger Pain

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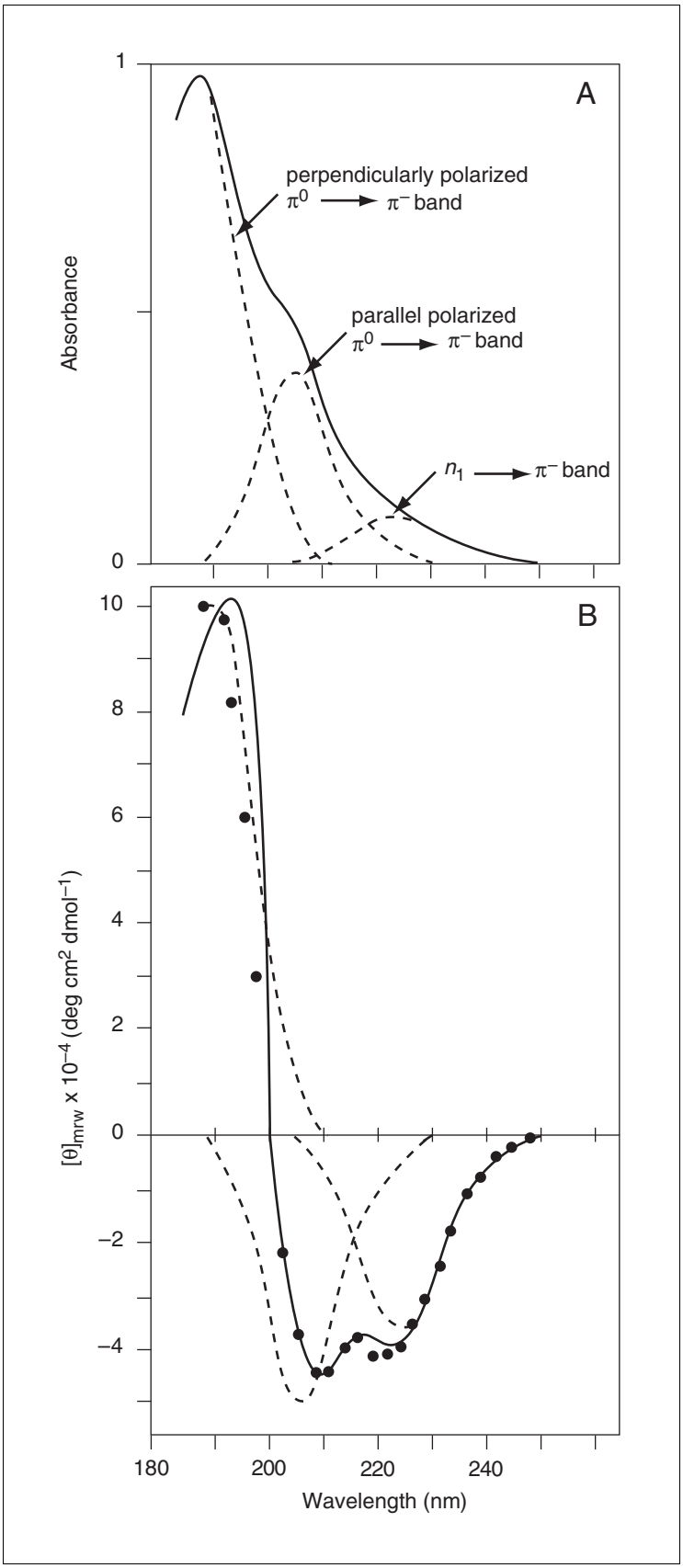

Figure B3.5.1 Relation between absorbance and circular dichroism for a polypeptide chain in the helical conformation. (**A**) The absorbance spectrum of poly-γ-methyl-L-glutamate in the α-helical form, with an assignment of its three constituent transitions. (**B**) The far-UV CD spectrum of the same compound, deconvoluted on the assumption that the shapes of the absorbance and dichroic bands are directly related (broken lines). The solid line is the observed spectrum and the filled circles the sum of the three bands. In general, the sign of a CD band may be either positive or negative, and its intensity does not necessarily follow that of the absorbance band. (From Holzwarth and Doty, 1965.)

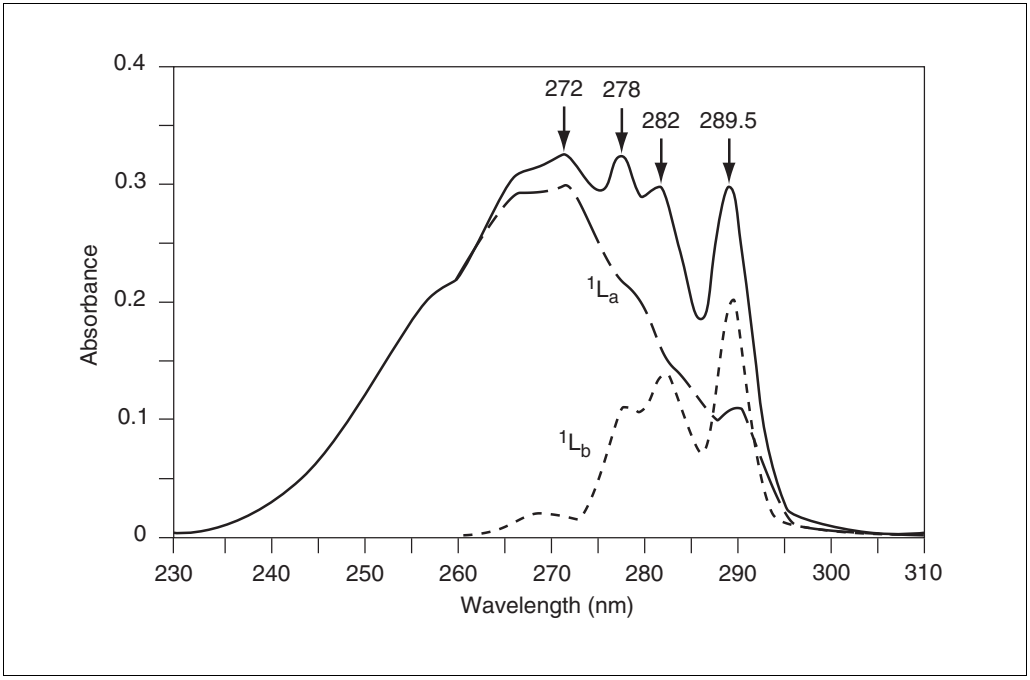

Figure B3.5.2 The two near-UV absorbance transitions for tryptophan. The near-UV absorption spectrum for *N*-stearyl tryptophan *n*-hexyl ester dissolved in methylcyclohexane at 24°C (solid line) has been resolved into the ^1L_a and ^1L_b bands. (Drawn from Strickland, 1974.)

in an optically symmetrical environment will normally absorb the two components equally so that, when recombined after passing through a solution of the chromophore, they result once again in radiation oscillating in a single plane. A chromophore situated in an optically asymmetric environment, however, will absorb each of the two components to a different extent, the difference being ΔA. When recombined, the resultant vector describes an ellipse (Fig. B3.5.3B), the ratio of whose major and minor axes determines the **ellipticity.** The value of ΔA, and hence that of ellipticity, can be positive or negative depending on the nature of the asymmetric environment. This is exemplified by the contribution of the tyrosines in IL-1β (Fig. B3.5.4A) and of the tyrosines, tryptophans, and heme in soybean peroxidase (Fig. B3.5.5).

It is a general consequence of the above principles that CD spectra of molecules in solution are located in the same wavelength region as their absorption bands. For proteins this means the far-UV and near-UV regions, as well as regions extending into the visible and near infrared. These regions have their origin in and provide information about the polypeptide backbone and its conformation (far-UV; Fig. B3.5.6), the aromatic amino acid residues and their environments (near-UV; Fig. B3.5.5), and bound ligands such as heme and cofactors (visible and near infrared). Experimentally, the near-UV, visible, and near-infrared regions can be treated together and the protocols in this unit will therefore refer explicitly only to the collection of far- and near-UV spectra.

In both the far- and near-UV regions, CD spectra can be used empirically as "fingerprints" of a particular protein, with the spectrum resulting from the aromatic residues being rather more specific and hence diagnostic. The far-UV spectra, however, can provide information about the protein conformation in terms of its secondary structure. As for fluorescence spectroscopy or any spectroscopic method, the sample needs to be chemically pure and homogeneous.

The output of CD spectrometers is in two alternative but related types of unit. The difference in absorbance with respect to left- and right-handed circularly polarized

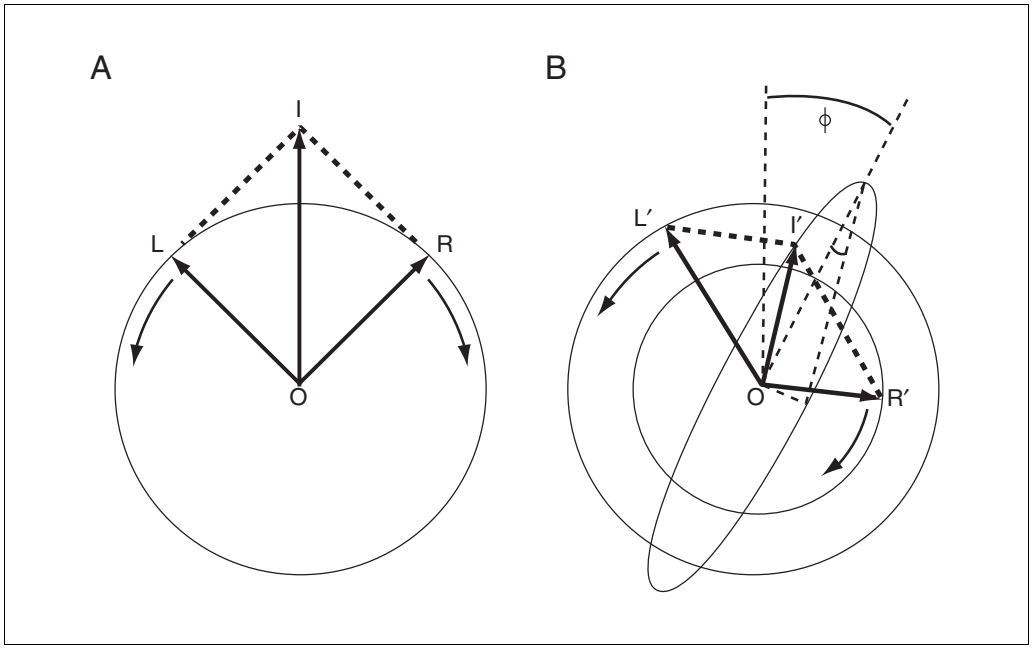

Figure B3.5.3 The relation of ellipticity to the differential absorption of circularly polarized radiation. The oscillating radiation sine wave, **OI**, is proceeding out of the plane of the paper towards the viewer. (**A**) Plane-polarized radiation is made up of left- and right-handed circularly polarized components, **OL** and **OR,** respectively. Absorption by a chromophore in a nonchiral environment results in an equal reduction in intensity of each component, whose resultant is a vector oscillating only in the vertical plane—i.e., plane-polarized radiation. (**B**) Interaction of the radiation with a chiral chromophore leads to unequal absorption, so that combination of the emerging vectors, **OL′** and **OR′**, leads to a resultant that describes an elliptical path as it progresses out of the plane of the paper. The ratio of the major and minor axes of the ellipse is expressed by tan θ, thus defining ellipticity. The major axis of the ellipse makes an angle (ϕ) with the original plane, which defines the optical rotation. This figure thus demonstrates the close relation between optical rotation and circular dichroism.

radiation ($A_L - A_R = \Delta A$) is measured in absorbance units; ellipticity (θ) is measured in millidegrees (mdeg). These units are related by the expression $\theta = 33{,}000 \times \Delta A$ (see Fig. B3.5.3A).

Ellipticity is usually used for far-UV measurements. Because essentially every residue in a protein is associated with a peptide bond, spectra in this region can be directly compared for different proteins by calculating the mean residue ellipticity,

$$[\theta]_{mrw} = \frac{\theta \times M_{mrw}}{10 \times c \times l}$$

where c is the protein concentration in mg/ml, l is the cell path length in cm, and M_{mrw} is the mean residue molecular weight—i.e., protein formula weight/number of residues. The dimensions of $[\theta]_{mrw}$ are deg cm^2/dmol, and the quantity is independent of molecular weight. M_{mrw} is in the range 110 to 115 for most proteins.

For example, if the ellipticity at 209 nm using a 1-mm path-length cell for a solution containing 0.361 mg/ml protein is −61.7 mdeg, and the mean residue molecular weight of the protein is 110, then $[\theta]_{mrw} = -18.8 \times 10^3$ deg cm^2/dmol.

Determining the CD Spectrum of a Protein

For small molecules, such as aromatic amino acids and model compounds, the molar difference absorption coefficient is frequently used. By analogy with absorbance spec-

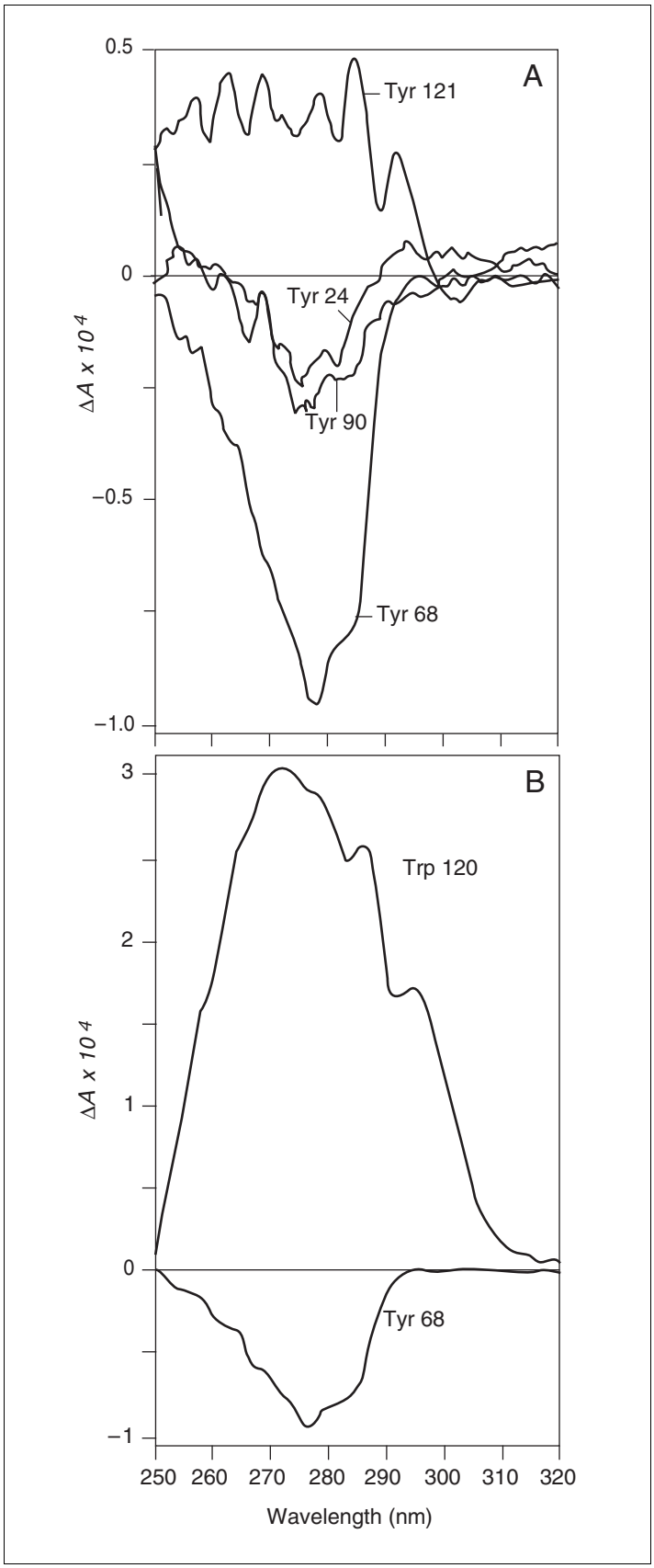

Figure B3.5.4 The contributions of different aromatic residues in a folded protein to circular dichroism. Interleukin 1β contains one tryptophan and four tyrosine residues. Their individual contributions have been resolved using site-directed mutagenesis. (**A**) The near-UV CD spectra of the tyrosine residues show that, although Tyr 68 makes a significant contribution to the negative ellipticity, indicating its buried, asymmetric environment, the contributions resulting from residues 24 and 90 are little different from that of free tyrosine. The derived spectrum of Tyr 121 is interesting in that it clearly contains a contribution arising from tryptophan (Fig. B3.5.2), indicating its close interaction with Trp 120. (**B**) The contribution from the tryptophan is seen to dominate, reflecting its nonpolar environment. The intensity of the protein spectrum is less intense than it might otherwise be, because of the opposing signs of the individual spectra. Spectra were measured using a 1-nm bandwidth (Craig et al., 1989).

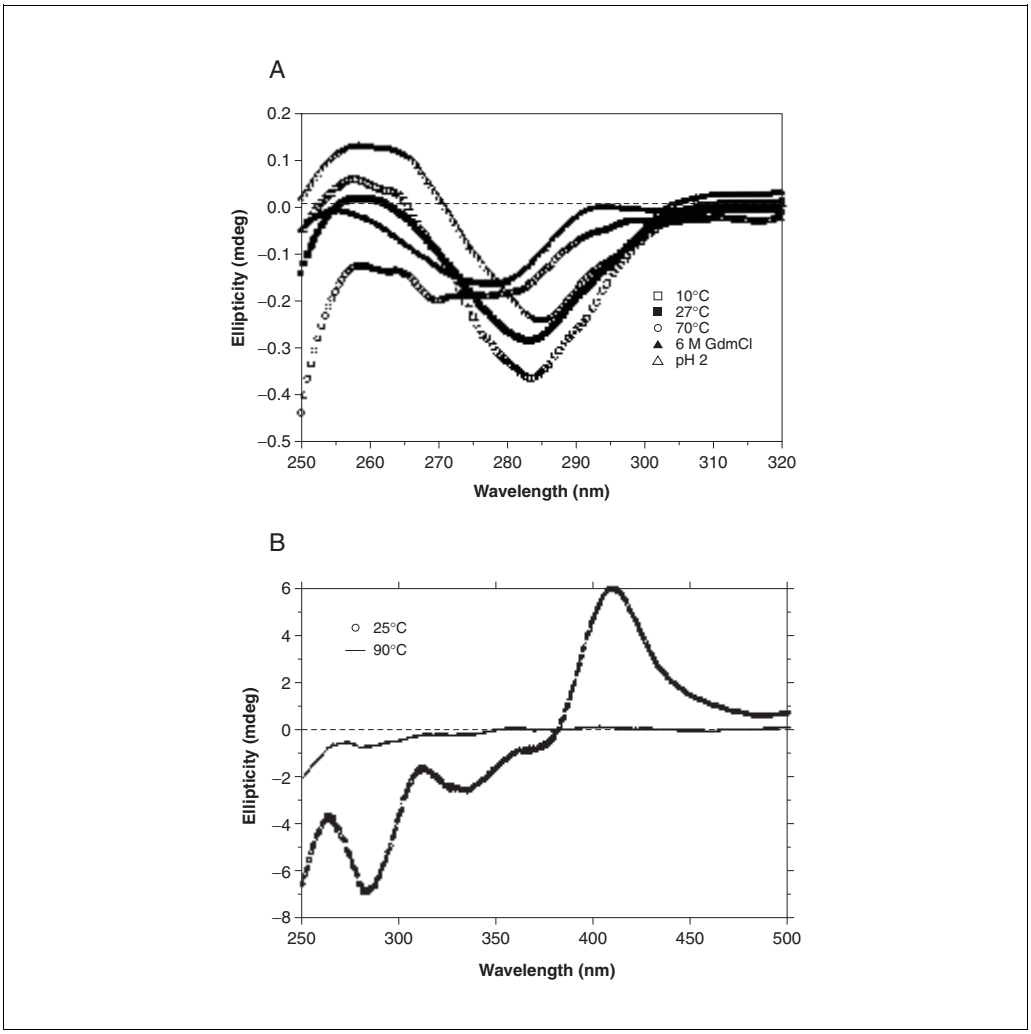

Figure B3.5.5 Near-UV CD spectra. (**A**) Bovine α_{s1}-casein peptide under a variety of conditions (data from Alaimo et al., 1999). Peptide concentration 0.631 mg/ml in 2 mM PIPES, 4 mM KCl, pH 6.75; scan rate 40 sec/nm; path length 10 mm; bandwidth 1.5 nm. The loss of aromatic dichroism with increasing temperature indicates denaturation, which is, however, not complete at 70°C or in 6 M guanidine hydrochloride. The shift in maximum wavelength indicates loss of tryptophan asymmetry, but less so of tyrosine. (**B**) Seed coat soybean peroxidase under native and denaturing conditions (data from Kamal and Behere, 2002). Protein concentration 15 μM and path length 10 mm. The negative aromatic band centered around 280 nm and the Soret band around 410 nm both disappear at 90°C, indicating the loss of net conformational asymmetry of the aromatic and heme chromophores.

troscopy, this quantity is given by $\Delta\varepsilon = \Delta A/(c \times l)$, where c is the concentration in mol/liter, l the path length in cm and $\Delta\varepsilon$ has the units liters/mol/cm (or cm^2/mmol). However, in the case of proteins, near-UV CD spectra are made up of contributions from tryptophan, tyrosine, and phenylalanine residues—the content of all of which vary from protein to protein. It is therefore confusing—and achieves no useful end—to present results in terms of mean residue ellipticity. Instead, dichroism should be presented as values of ΔA or ellipticity. For a given protein, different samples or mutants may be compared by calculating $\Delta A/(c \times l)$, with c in mg/ml.

Owing to the variety of units found in the literature and in software—e.g., mdeg and deg for ellipticity, and mm, cm or dm for path length—it is necessary to take care that experimental parameters are entered in the units requested by the machine software.

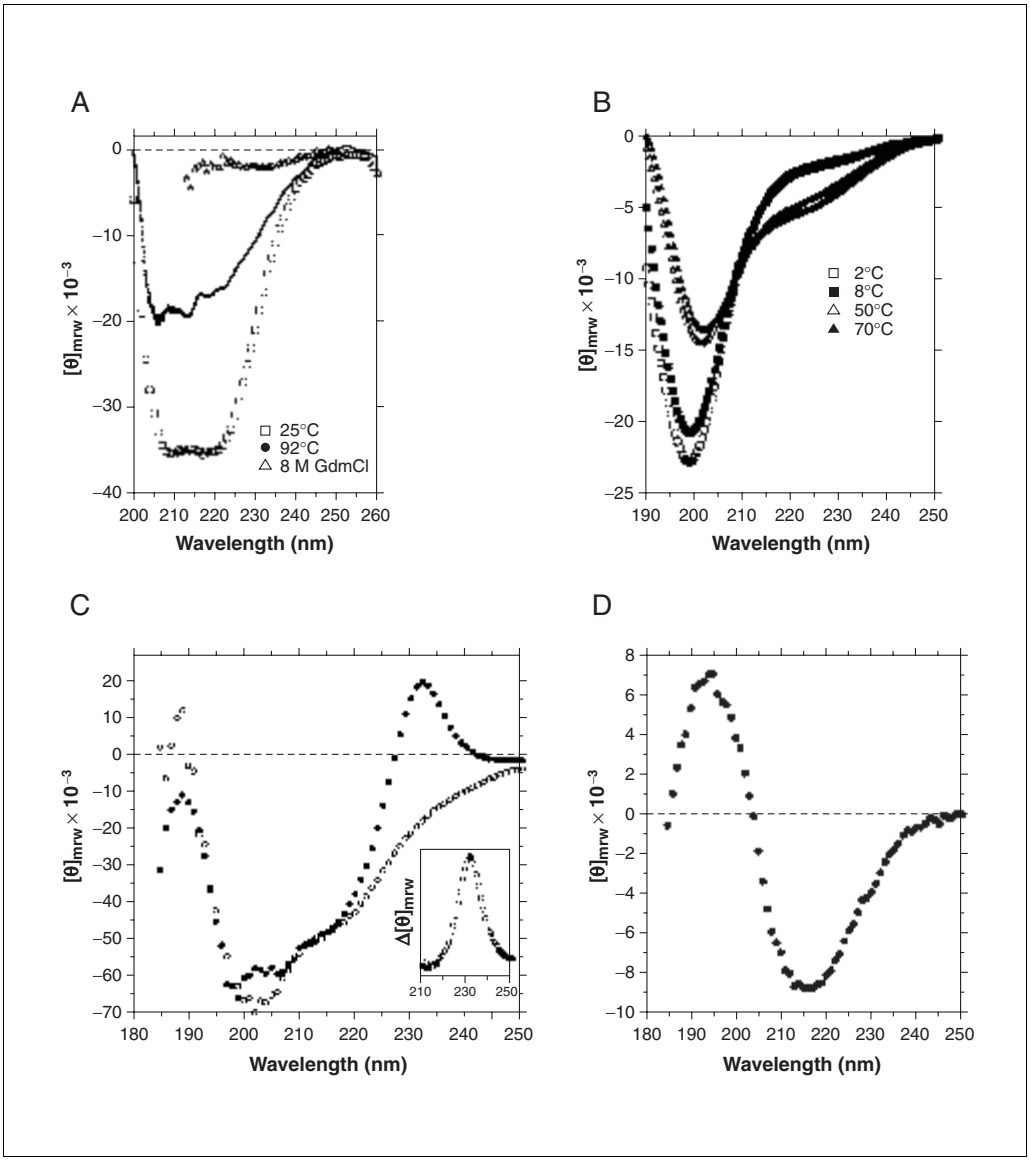

Figure B3.5.6 Far-UV CD spectra. (**A**) Seed coat soybean peroxidase under native and denaturing conditions (data from Kamal and Behere, 2002). Protein concentration 6 μM and path length 1 mm. The troughs at 208 and 222 nm are characteristic of a high content of α-helix. Unlike the aromatic and heme residues (Fig. B3.5.5B), this secondary structure is not completely disrupted at 90°C, but requires a strong chemical denaturant. The cut-off in measurements in 8 M GdmCl is due to the strong absorbance of the latter in the far UV, making it impossible to make measurements at lower wavelengths. (**B**) Bovine β-casein as a function of temperature (data from Farrell et al., 2001). Protein concentrations in the range of 0.18 mg/ml; path length 0.50 mm; bandwidth 1.5 nm. Note the minimum at 199 nm whose intensity and wavelength shift at higher temperatures, suggesting low cooperativity of the structure in solution. Note also the appearance at higher temperatures of an apparent contribution from aromatic asymmetry centered on ~226 nm. (**C**) Clitocypin—a protease inhibitor from mushroom (data from Kidric et al., 2002). Protein concentration 0.1 mg/ml in 50 mM phosphate, pH 6.6; path length 1 mm; bandwidth 2 nm; scan step 1 nm; dwell time at each step 20 sec; four repeats. Note the minimum around 200 nm and peak at 189 nm due to the high content of β structure. The intense peak at 231 nm, which disappears on thermal denaturation, is due to an aromatic contribution, probably due to a tryptophan-tryptophan interaction in the native protein. The symmetry of this peak (inset) suggests a single chromophore transition. (**D**) Immunoglobulin G. Protein concentration 0.03 mg/ml; path length 1 mm; bandwidth 1 nm; scan step 1 nm; dwell time at each step 5 sec (i.e., scan speed 12 nm/min). The minimum at 216 nm and peak at 194 nm are characteristic of the classical β-structure CD spectrum, in contrast to those of casein and clitocypin.

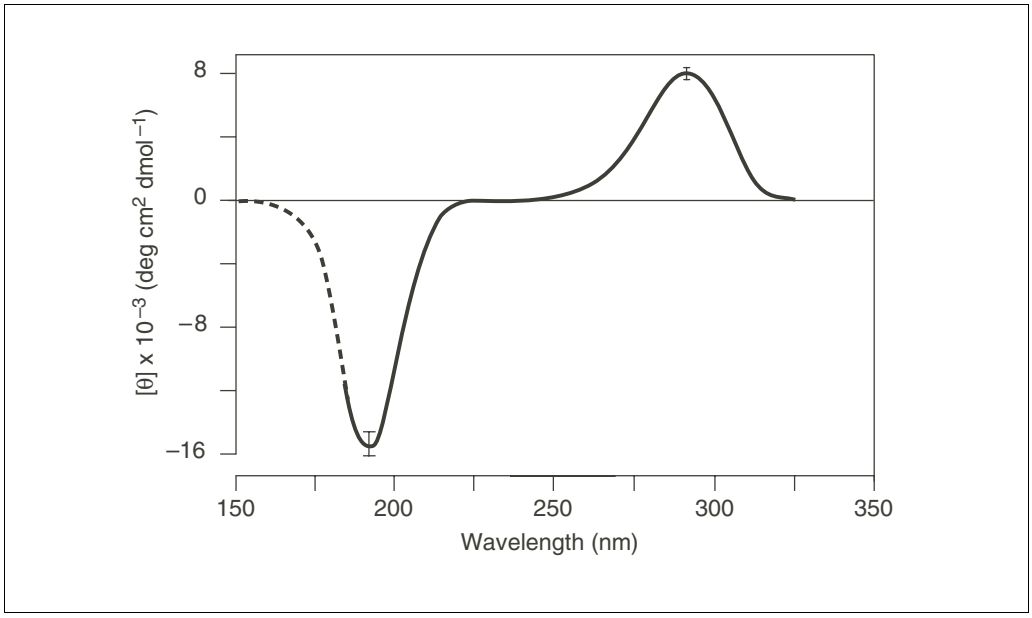

Figure B3.5.7 CD spectrum of D-(+)-10-camphorsulfonic acid (CSA) in water. The vertical bars represent variations of ±1.5% and ± 5%. The broken line represents the extrapolation of a gaussian band. Commercial CSA was twice recrystallized. (From Chen and Yang, 1977.)

The Strategic Planning section deals with considerations regarding instrumentation and reagents for CD spectrometry. The Basic Protocol outlines the steps in recording a CD spectrum. The two support protocols explain the interpretation of CD spectra—Support Protocol 1 deals with near-UV spectra and Support Protocol 2 with far-UV spectra.

STRATEGIC PLANNING

CD Spectrometer

CD spectra are measured using a CD spectrometer, of which there are three different makes on the market (AVIV Associates, Jasco, and Applied Photophysics; see *SUPPLIERS APPENDIX*). The first two are classical spectrometers with some capability for studying fast reactions by CD, while the third is specifically designed for fast-reaction CD. These instruments are fitted for control, data collection, and data handling by computer. The principles of how they function are generally outlined in the manufacturers' manuals and a good general account is given in Bayley (1980). A supply of high-purity nitrogen is essential to displace oxygen in order to avoid degradation of the mirrors by ozone generated by the high-power xenon sources, as well as to reduce absorbance from oxygen bands below 200 nm (see discussion of Nitrogen Supply, below). A satisfactory means of thermostatting cells is essential for work with proteins (see discussion of Cells, below), using a water-circulation system or the more convenient Peltier thermostat.

The CD spectrometer is usually required to work near the limits of sensitivity—e.g., reading ΔA values of $\leq 10^{-4}$ at a total absorbance of 1. Thus, particular care needs to be taken with cleanliness and orientation of cells and with settings of scan rate, time constant, and bandwidth. It is also important, especially when recording far-UV spectra, that the lamp is not old and that the mirrors are not clouded from radiation and traces of ozone. Because the spectrometer is a single-beam instrument, it is essential always to watch carefully for evidence of instrumental drift during measurements of sample and baseline.

Calibration of the Spectrometer

Depending on the make of the spectrometer, wavelength calibration may require the use of a mercury lamp and the services of an engineer, or the use of a holmium oxide solution with a simple computer-operated adjustment. It is unlikely to vary significantly during the lifetime of a xenon lamp. Calibration of ellipticity, on the other hand, must be carried out regularly—at least monthly, if the machine is in regular use—using a purified compound of known absorbance and ellipticity. D-(+)-10-Camphorsulfonic acid (CSA) is the substance of choice (Fig. B3.5.7), but care must be taken to purify it from optically active impurities. Crystallization from ethyl acetate is recommended. CSA is hygroscopic; hence, although a stable monohydrate can be prepared (Yang et al., 1986), the concentration of the aqueous solution (~1 mg/ml) should be determined from $A_{285}^{1\ mg/ml} = 0.149$ using a high-quality spectrophotometer. Calibration of the spectrometer is carried out according to the maker's instructions, using $\theta = 33.5$ mdeg at 290.5 nm for a solution of 1.00 mg/ml anhydrous CSA in a 1-mm path-length cell. CSA exhibits a second peak at 192.5 nm where $\theta \approx -67.0$ mdeg for the same solution. If the value of the ratio of ellipticity at the two wavelengths falls below 2.0, this indicates a loss of performance of the spectrometer. Epiandrosterone, at 304 nm, and D-(−)-pantoyllactone, at 219 nm, have also been used for calibration (Schmid, 1989). The peaks of the spectra scanned for calibration are sufficiently sharp to provide a check on the wavelength calibration.

Cells

Choice of cells

Quartz windows can exhibit dichroism, which is due to strain either remaining after the annealing process during manufacture or induced by distortion caused by heating or pressure. It is important for measurement of CD that the optical faces of the quartz QS cuvettes have low strain and exhibit low dichroism. In the near-UV region—where path lengths ≥5 mm are required—rectangular cells, in their standard, semimicro, or micro forms, are normally used. These are not only more economical with regard to protein than the cylindrical type, but allow mixing to be carried out in the cell. Testing a number of rectangular cells in the CD spectrometer usually yields one or two with low dichroism; it is also possible to purchase cells with certificates of low dichroism. Careful observation of the height of the light beam at the cell allows the minimum volume of solution to be used—usually <1 ml in a 10 × 10–mm cell. Because standard quartz (Suprasil) cells are constructed from two different types of glass, differential expansion of the faces with temperature leads to a significant change in cell dichroism. At least for temperature-dependence experiments, it is recommended to use cells with all four faces made from fused quartz. Semimicro cuvettes with 1-cm path length are available; these require ~300 µl of solution.

For far-UV CD spectra, short-path-length cells—from 1 mm down to 0.01 mm—are necessary because of the higher absorbance and ellipticity in this region. Demountable cells, cylindrical or rectangular, of this size are easily cleaned, but require practice to fill without allowing evaporation and may be subject to solvent loss over long periods of time. Such leakage can be minimized by the use of Parafilm on the edges of the cells. Quartz-jacketed cylindrical cells with two filling holes, available from Hellma (see *SUPPLIERS APPENDIX*), provide the best economy of protein, temperature control, and reproducibility. The path length of cells should be checked by absorbance measurements. A solution of 300 mg/liter of potassium dichromate in 0.05 M KOH gives an A_{280} of 0.705 in a 1-mm cell.

Preparation and handling of cells

Cells must be reproducibly free from external and internal contamination. Not only will contamination make a contribution to the measured ellipticity, but dirty surfaces cause bubbles to be trapped in short-path-length cells, leading to additional artifacts. Cleaning should always be repeated if there are any signs of "tailing." The short-path-length cells, being inaccessible to mechanical cleaning, have to rely on effective soaking and rinsing. Cuvettes must be thoroughly and reproducibly cleaned by soaking in a proprietary cuvette cleaning fluid such as Hellmanex II (Hellma), an alkaline liquid concentrate, or RBS-35 Detergent Concentrate (Pierce), following the makers' instructions. The makers of Hellma cuvettes caution against the use of ultrasonics as a means of boosting cleaning efficiency. 50% nitric acid is also effective, but requires care and a fume hood. Detergents frequently contain fluorescent material and, if used, should be removed from cuvettes with acid (2M HCl) followed by water. After the final rinse with copious amounts of high-quality distilled water, the cells are best dried using suction through a Pasteur pipet protected with a small length of fine plastic tubing. This is safer than using acetone or alcohol, which can leach out fats from fingers and deposit them on the cuvette. The faces of cuvettes must not be touched after cleaning. Filling, emptying, and rinsing between measurements of spectra must be done using pipets, to avoid spillage onto the outer faces. A Pasteur pipet protected with fine plastic tubing is satisfactory. The baseline scans, with buffer in the cell, must be examined as a constant check on the reproducibility of the state of the cell.

Buffer Solutions

Buffers must be optically inactive and have the lowest possible absorbance in the desired spectral region. Most buffers in common use are essentially transparent above 230 nm, which is fine for near-UV CD. For far-UV CD using a 1-mm pathlength cell, 0.01 M sodium phosphate buffer (*APPENDIX 2A*), is generally acceptable as a solvent for most purposes, and will still have a reasonably low absorbance between 190 and 185 nm—the lower the pH, the more transparent the buffer. NaCl should be avoided in the far-UV range. Solutions of 0.01 M $NaClO_4$, NaF, KF, or boric acid are all transparent down to the present range of commercial instruments, but are not frequently used except for vacuum UV measurements. The absorbance of 20 mM Tris·Cl is acceptable down to 190 nm. Buffers in common use, together with their absorbances, are listed by Schmid (1989). Urea or guanidine·HCl solutions (high-purity spectroscopic grades must be used) absorb very strongly below 220 nm; even in the shortest-path-length cells, they are opaque below 205 nm.

Bubbles form readily in the cell and can distort the CD. It is important therefore to degas the buffer before filling the cell by applying vacuum in a suitable container, such as a Buchner funnel, and swirling.

Clarification of Solutions

Protein solutions must be freed from the turbidity that results from dust and aggregated protein. Turbidity will reduce the sensitivity of the measurements and can also cause artifacts in both the intensity and the shape of the CD spectrum, particularly in the far-UV (Swords and Wallace, 1993). Many proteins can be filtered, usually as a stock solution, using a 0.22-μm filter (ideally of the grade special for proteins) fitted to a syringe. If the protein is adsorbed by filters, the solution may be clarified by centrifugation; 10 min in a refrigerated microcentrifuge is convenient and usually effective. Note that the protein concentration must be measured *after* clarification. Buffer solutions are filtered on a larger scale, using a 0.22-μm filter.

It is essential to monitor clarification if artifacts are to be avoided. Indicators of adequate

clarification that should be adopted for spectroscopic characterization are available from the procedures basic to the measurement of CD. Determination of protein concentration requires an absorbance spectrum to be recorded on a good quality spectrophotometer from 240 to 350 nm. Aromatic amino acid residues do not absorb above 320 nm, so the spectrum between 320 and 350 nm should be only marginally above baseline. The presence of turbidity will result in a sloping spectrum in this region. From the absorbance spectrum obtained in this manner, the ratio A_{max}/A_{min} is calculated, where A_{min} is the absorbance in the trough in the region of 240 to 250 nm. This ratio will be specific for a given protein and should be measured for a sample that has been carefully and reproducibly clarified. Because of the strong wavelength dependence of scattering, the ratio is a very sensitive indicator, decreasing sharply with turbidity, and can be used as a semiquantitative check on the clarity of subsequent solutions of the protein.

For membrane proteins in membrane fragments, the intrinsic turbidity offers special problems, which should be addressed by instrumental modifications. A useful discussion of the problems involved can be found in Swords and Wallace (1993).

Nitrogen Supply

Oxygen-free nitrogen can be obtained from cylinders, but must be filtered to remove oil contaminants. Liquid nitrogen provides a better supply of gas, but impurities accumulate at the bottom of the Dewar flask, so that the bottom 10% should not be used (Johnson, 1990). Accessories to monitor and purify nitrogen gas are available commercially.

RECORDING A CD SPECTRUM

BASIC PROTOCOL

Simple comparison of the CD spectra of a protein in buffer and in 6 M guanidine·HCl will immediately tell whether the protein has achieved some degree of folding. However, for the purpose of determining whether a sample of recombinant protein is correctly folded, the CD spectrum can provide a complex fingerprint that can be compared with that of a specimen that has been well authenticated as being native and functional. This constitutes an empirical, but powerful means of characterization, with the near-UV spectrum usually being more sensitive than its far-UV counterpart. There are, for example, many well-documented cases where partially unfolded proteins exhibit a major change in the aromatic region accompanied by relatively minor changes in the far-UV CD, the classical molten globule being an important example (Kuwajima and Arai, 2000). For these reasons, it is important to obtain good quality near-UV spectra, paying particular attention to details of any fine structure that may exist.

Table B3.5.1 Rough Guide for Scanning a Protein Solution with A_{280} = ~1

	Far-UV	Near-UV
Cell path length (cm)	0.01–0.05	1
Wavelength (nm)	250–180	340–250
Bandwidth (nm)	0.5–1	0.2–0.5
Averaging time (sec)	1	1–5
or		
Time constant (sec)	2–8	8
Repeat scans	1–2	2–4
Step size (nm)	0.5–1	0.2–0.5
Resulting time per scan (min)	70–140	3–37

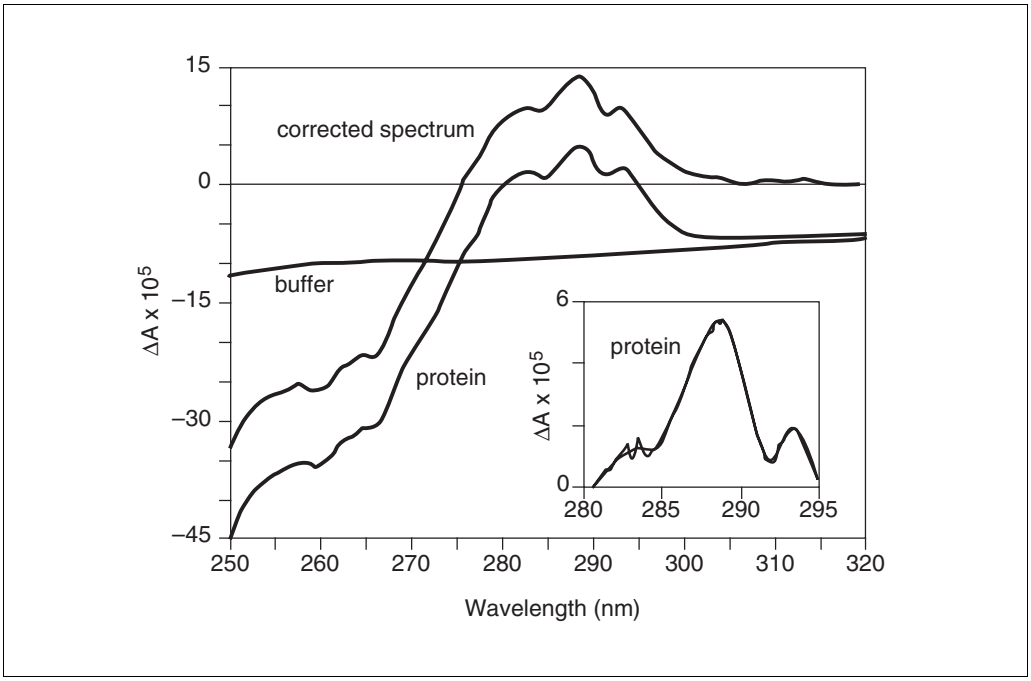

Figure B3.5.8 Obtaining the corrected near-UV CD spectrum for hen egg white lysozyme. The protein and baseline spectra were collected using a 10-mm cylindrical cell and 0.5 mg/ml protein in 0.067 M phosphate buffer, pH 6.0. Instrument settings were 1-nm bandwidth, 0.2-nm step size, scan speed 2 nm/min, time constant 8 sec (scan speed × time constant = 0.27 nm). Protein solution and buffer were scanned once each. The spectra were smoothed, a sample of the fit being shown in the inset. Reproducibility of the instrument and of the state of the cell are demonstrated by the coincidence of the ellipticity above 300 nm. The corrected spectrum was obtained by subtraction, using the instrument software.

Materials *(also see Strategic Planning)*

Buffer solution
Clarified protein solution for analysis
Nitrogen supply
CD spectrometer (calibrated) and cells

1. Set up the CD spectrometer by purging the optics with nitrogen in the manner designated in the manual, turning on the cooling water, and finally switching on the lamp. Allow to warm up for the recommended period of time, usually 30 min. Regulate the thermostat system to the desired temperature.

   Particular attention must be paid to the procedure in the manual for switching on and warming up the spectrometer. Failure to purge with nitrogen can lead to rapid and irreversible degradation of the optics.

   CD spectra, particularly in the near-UV (see Support Protocol 1) reflect the dynamics of the chromophore and may therefore show dependence on temperature. It is important to stabilize the temperature reproducibly. Accurate temperature control is particularly important in denaturation experiments to determine protein stability.

2. Enter the settings required for the scan.

   Careful optimization of the settings is necessary to achieve the lowest-noise spectra consistent with total time spent in data collection. To check that optimal settings have been achieved, a small sample region of the spectrum can be scanned.

   Table B3.5.1 is a rough guide for scanning a protein solution with A_{280} = ~1. Settings will differ according to the sample and the spectrometer.

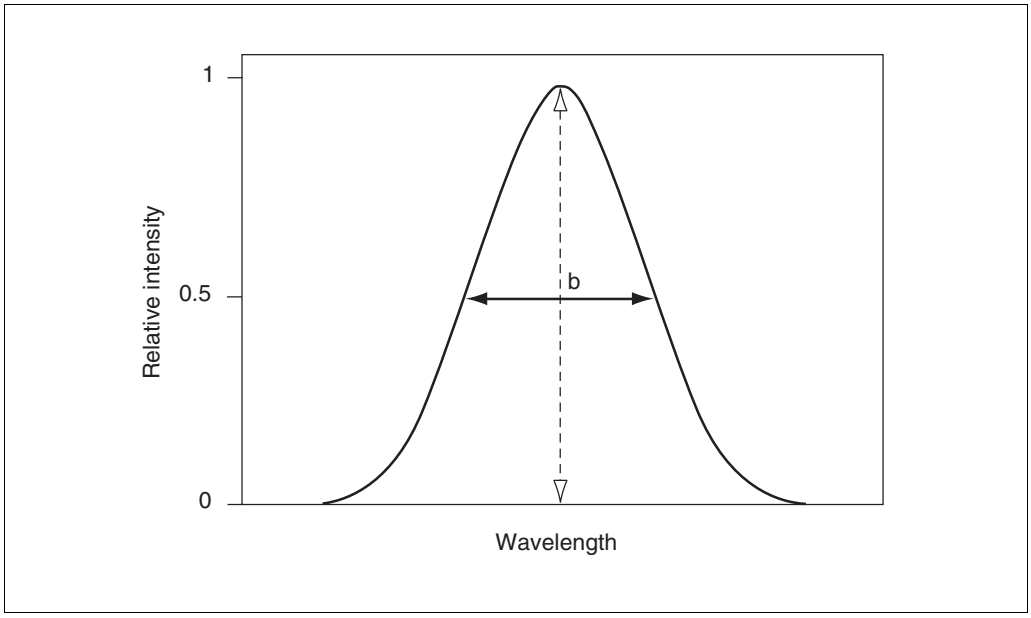

Figure B3.5.9 The distribution of intensity of radiation passing through the sample cell. Radiation emerging from a monochromator set at a wavelength corresponding to the peak maximum will contain components at other wavelengths, with intensities described by the curve. The bandwidth (b) is the width of the distribution curve at half the peak height. Its magnitude will depend on the exit slit width. The setting of the slit width is usually programmed to change during scanning, so as to give a constant bandwidth and hence constant spectral resolution.

a. Set wavelength.

For near-UV spectra, the scan should be from 240 to 340 nm, in order to cover contributions from tryptophan, tyrosine and phenylalanine. Although little or no dichroism will be found above 320 nm, it is advisable to extend the scan to 340 nm to ensure that both sample and baseline scans coincide at zero ellipticity in the region where there is no dichroism. This provides assurance that the cell and buffer are optically identical for the two scans (see Fig. B3.5.8).

For far-UV spectra, it is usual to scan between 250 nm—where the ellipticities of the baseline and sample effectively coincide, given the scale of ellipticity being used—and as near to 180 nm as the absorbance of the solution will permit. The lower limit may be dictated by the absorbance of the sample or buffer, as indicated by the value of the dynode voltage (see step 2e).

b. Set bandwidth.

The radiation passing through the sample comprises a spread of intensities distributed around the selected wavelength (Fig. B3.5.9). The bandwidth (in nm) of this spread is determined by the spectrometer slit width (in mm) and is the width of the distribution at half the maximum intensity. For proteins exhibiting broad CD bands, including most far-UV and many near-UV spectra, a wide setting (2 nm) will have little effect on the shape or intensity of the spectrum. For bands with fine structure, including some near-UV spectra, a more narrow bandwidth is necessary to avoid smoothing of narrow peaks and reduction in peak intensity. The largest bandwidth that causes no distortion of the spectrum should be used, in order to maximize the signal-to-noise ratio. To optimize the slit-width setting, preliminary scans should be made at a series of slit widths over a critical region of the spectrum.

c. Set averaging time or time constant, scan speed, and number of accumulations.

The time constant determines the time that the spectrometer takes to respond to changes in signal. The reason for setting it is to obtain the best signal-to-noise ratio consistent with the time spent scanning. When the signal is strong, the time constant need only be small. The appropriate time constant for a signal of 500 mdeg is 0.25, that for a signal of 100

mdeg is 0.25 to 0.5, that for a signal of 10 mdeg is 0.5 to 0.8, and that for a signal of 2 mdeg is 2 to 30.

The faster the scan speed (with economies in operator time), the smaller the time constant must be in order to allow the instrument to respond fully to changes in ellipticity. However, less time is then available for collecting data at a given wavelength, so the noise is greater. Scan speed and time constant are thus interdependent, and their product should not exceed 0.33 nm. For example, a time constant of 2 sec is recommended for a scan speed of 10 nm/min. Some instruments, in the wavelength scan mode, specify an averaging time, which takes the place of a time constant setting; this is simply the time spent collecting data at each wavelength.

The signal-to-noise ratio can be enhanced either by increasing the time constant or averaging time or by accumulation of a number of scans. The latter technique is preferable to using long scan times because the effect of instrument drift will be to distort the spectrum from a single long scan, whereas the average of quicker, repeat scans will lead to a more uniformly shifted spectrum. The latter can then be corrected using the average of buffer baselines measured before and after the sample scans.

The problem of optimizing the signal-to-noise ratio is greater in the case of samples possessing a low ellipticity. An all-β protein, for example, will demand more repeat scans than an all-helix protein at the same concentration.

There are realistic time limits to the degree of enhancement of the signal-to-noise ratio, as the latter is proportional to the square root of both the time constant and the number of scans (see Yang et al., 1986).

d. Set step size.

The step size (in nm) between successive data-collection wavelengths, together with the scan speed, defines the number of data points collected per nm. Spectra with more fine structure require higher resolution. For far-UV spectra, which are smooth and relatively featureless, a step size of 0.5 nm is adequate. For some near-UV spectra, but not all, a resolution of 0.2 or 0.1 nm is advisable. For a given scan speed, the greater resolution gained by using a small step size will result in more noise, so it is more economical of time to use as large a step size as possible.

e. Note dynode voltage.

Dynode voltage cannot be preset, but its value can be controlled within limits by adjusting the protein concentration and/or cell path length, so it is appropriate to consider it here. The photomultiplier detector operates with a high voltage in the region of 1000 V across the anode and cathode. When light falls on the photosensitive cathode, current flows and the potential difference is reduced. Readings of the dynode voltage thus give a measure of the intensity of light passing through the sample. Higher values indicate low levels of light (high sample absorbance) with consequent decrease in signal-to noise-ratio; very high values indicate incorrect readings. Values of dynode voltage should always be noted during a scan in order to assess the reliability of the ellipticity measurements. Most instruments record this parameter as a matter of course. Special care must be exercised in the far-UV region, where sample and buffer absorbance can rise sharply with decreasing wavelength.

3. Fill the cuvette and place it in the cell holder, making sure that the orientation is reproducible both longitudinally and rotationally. Mark the cell to allow reproducible orientation in the light beam.

Techniques for filling the cell will depend on the type and path length of cell being used. It is essential in all cases to avoid the presence of small bubbles, which can easily be trapped at the optical face, especially with shorter-path-length cells. Even slight contamination of the faces will make this difficult, necessitating frequent cleaning of the cell after contact with certain proteins and with buffer components such as guanidine·HCl.

For rectangular-type cuvettes, the volumes of solution required are approximately 2.5 ml (for 1-cm standard), 1 ml (for 1-cm semimicro), 300 µl (for 0.1-cm standard) and 25 µl

(for 0.01-cm demountable). Volumes for cylindrical-type cells are somewhat larger, depending on the type.

Choice of path length will depend on the available concentration of the protein solution. If the absorbance of the buffer is unavoidably high, it will be advantageous to use a shorter path length and a higher protein concentration. As a general guide, for a protein solution with A_{280} = 1, path lengths of 0.01 to 0.05 cm for the far-UV and 1 cm for the near-UV make suitable starting points.

Rectangular cells have the advantages over cylindrical cells of having smaller sample volume and better temperature control with available cell holders. Good temperature control with cylindrical cells requires the use of jacketed cells. For shorter (<0.1-cm) path lengths, where rectangular cells are currently demountable, the rigid cylindrical cells have the advantage of avoiding leakage over prolonged periods of time.

4. Scan the baseline using the same cell and buffer as required for the sample, as well as the same instrument settings.

Preliminary scans of the samples should be carried out to assess whether valid measurements can be made in the required wavelength region or whether it is necessary to adjust the sample concentration or the cell path length. The wavelength is lowered, noting the point at which the photomultiplier voltage exceeds the value corresponding to low light intensity—i.e., to an absorbance of 1 to 1.5. Experience will indicate the value above which noise becomes excessive. It is helpful, however, to get a more objective assessment by noting the voltage at a series of low wavelengths and then measuring the actual absorbance of the same cell using a quality spectrophotometer.

When recording spectra for a series of samples, buffer scans should be recorded at least twice (at the beginning and end of the series) in order to check for instrument drift, especially when the lamp is beginning to age.

A convenient procedure when scanning a number of samples is to scan air blanks—i.e., with no cell in the light path—at appropriate intervals between samples. This saves additional cell filling, washing, and drying operations. A single scan (S) of the cell containing buffer accompanied by a scan of the air blank then allows correction of all the protein spectra for solvent baseline, according to the expression:

$$(S_{protein} - S_{air}) - (S_{solvent} - S_{air}) = (S_{protein} - S_{solvent}).$$

Ideally, the buffer scan should be run before the protein sample, as this requires less rigorous washing of the cell between the two runs and hence better assurance that the optical surfaces will be identical for both. This, however, requires prior knowledge of the settings appropriate to the particular sample.

It is tempting to use water in place of buffer on the grounds that both are optically inactive and, for water, the cell needs only to be dried between scans. This is unwise, however, particularly for buffers with higher concentrations of solutes and hence higher refractive index. Because the light beam in most instruments is not parallel when passing through the cell, the area and position of the photomultiplier surface illuminated is likely to vary with the refractive index of the solution, which may give slightly different voltage readings. This may result in significant errors when the signal is low, as in the case of near-UV CD.

5. Remove the buffer solution, rinse the cell with water and dry, taking care not to contaminate or touch the outer surfaces. Refill the cell with the clarified sample solution and scan the sample using the same instrument settings as for the buffer.

Because washing, drying, and filling the cell takes time, it is advisable to work with two cells alternately (both with solvent blanks properly determined) when processing a number of samples.

The protein concentration should be as high as possible, consistent with the absorbance in the cell not exceeding 1.0 to 1.5, and must be accurately determined by UV absorbance. Values of mean residue ellipticity and secondary structure content results are both affected by uncertainties in protein concentration.

Table B3.5.2 Transitions of Aromatic Amino Acid Side Chains[a]

Amino acid	Electronic transitions	Peak wavelengths (nm)	$\Delta\varepsilon_{max}$ (M^{-1} cm^{-1})[b]
Phenylalanine	^1L_b	262, 268	±0.3
Tyrosine	^1L_a	far UV	
	^1L_b	277, 283	±2.0
		268, 289 (shoulders)	
Tryptophan	^1L_a	266	±3
		~295-303[c]	
	^1L_b	285, 292, 305[d]	±2.5

[a]Data from Strickland (1974).

[b]Estimated values for an isolated side chain.

[c]May be seen as a low-intensity shoulder or peak from a Trp in a nonpolar environment.

[d]Unusual; observed with, e.g., lysozyme.

In spectral regions such as the far-UV, where absorbance varies markedly with wavelength, the spectrum may have to be recorded in overlapping regions, at appropriate concentrations or cell path lengths.

A good test for the absence of artifacts that can arise at higher absorbances is to run a blank with a non–optically active molecule, such as 3-methylindole or a DL–amino acid, at the same absorbance as the test samples. The scan should be indistinguishable from the solvent blank.

6. Subtract the baseline from the sample scan.

The ellipticity of the spectrum corrected for baseline should be close to zero above 320 nm for near-UV and above 250 nm for far-UV spectra (see Fig. B3.5.6 and Fig. B3.5.8). Significant differences usually indicate problems with reproducibility of the buffer or cell and must be remedied for quantitative characterization of samples.

Depending on the level of noise, spectra and baselines can be smoothed using the instrument software. Smoothed and raw spectra should be compared directly by overlaying them on the computer screen in order to avoid the distortion that can arise from overenthusiastic smoothing.

SUPPORT PROTOCOL 1

INTERPRETATION OF NEAR-UV CD SPECTRA

CD spectra reflect, in different ways, the detailed conformational environment of individual chromophores in a protein. To the extent that the transitions for the different types of chromophore differ in wavelength, the spectra can be assigned to particular types of residue and, in the case of the peptide bond, to different conformations or types of secondary structure. A brief account of the interpretation of near- and far-UV spectra is therefore pertinent. A more detailed discussion can be found in Sreerama et al. (1999a).

Near-UV CD spectra provide evidence of tertiary structure and dynamics. The CD of chromophores absorbing in the near-UV region is associated with the π–π^* electronic transitions. Relevant transitions for the aromatic amino acids are listed in Table B3.5.2. Tryptophan, tyrosine and phenylalanine possess partial or complete planes of symmetry, so that their dichroism arises almost entirely as a result of interaction of the transitions with neighboring groups. Dipole-dipole interactions, especially with other aromatic rings and with the π–π^* transition of peptide bonds, make a major contribution to CD and are relatively long-range, being effective up to ~1 nm. Electric-magnetic coupling also has an influence on the CD, but is very short-range and makes a relatively minor contribution.

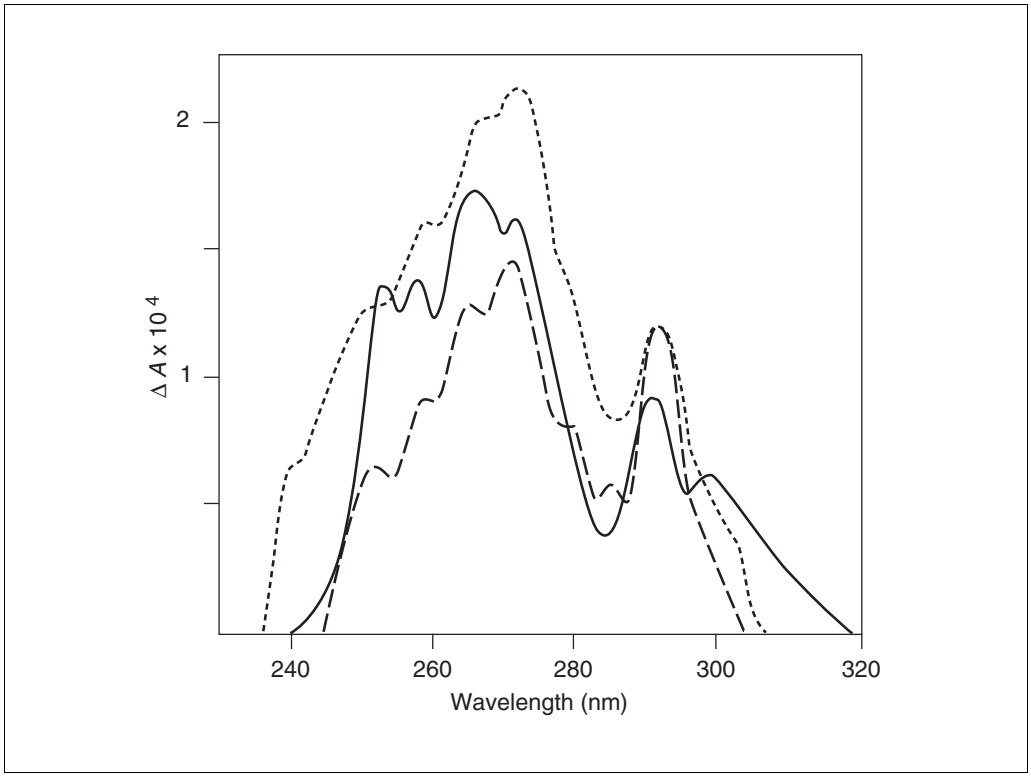

Figure B3.5.10 The near-UV CD spectra of cathepsin D. The solid line represents the porcine enzyme and the dashed line represents the bovine enzyme, each cleaved into two chains which remain associated; the dotted line represents the bovine intact enzyme. The spectra were recorded using 10-mm- and 20-mm-path-length cells, with enzyme concentrations of 0.8 to 0.4 g/liter. The far-UV CD and fluorescence spectra of the cleaved and intact enzymes are not significantly different (Pain et al., 1985).

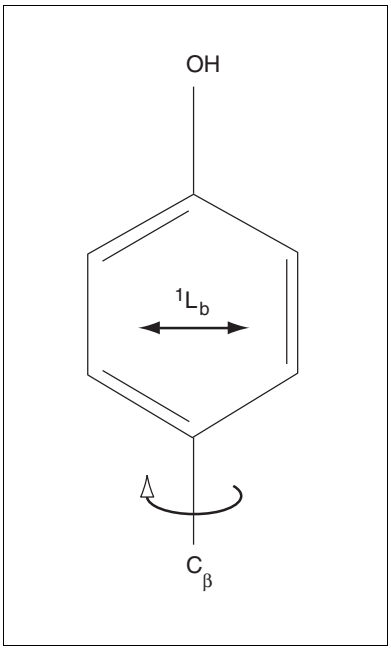

Figure B3.5.11 The near-UV absorbance transition dipole moment of the tyrosine side chain. The ^1L_b transition is seen to be perpendicular to the axis of rotation of the phenolic ring. Interactions of the transition with the environment, expressed as an enhanced CD band, will be sensitive to rotation of the side chain around this axis.

In contrast to the aromatic residues, the disulfide bond has no plane of symmetry and is, as a result, intrinsically optically active, particularly around a dihedral angle of 90°.

These interactions of the environment with transitions of aromatic residues result in dichroic bands that are either positive or negative so that statistically the more such residues there are, the less intense the resultant ellipticity is likely to be. An intense signal frequently indicates the presence of one particularly strong interaction that overrides other minor contributions—such as the strong band at 292 nm seen in aspartic proteinases (Fig. B3.5.10), and the contributions of the one tryptophan and four tyrosine residues to the CD spectrum of interleukin 1β (Fig. B3.5.4).

The ^1L_b transition for **phenylalanine** resembles that for benzene (Strickland, 1974), but the CD signal is of relatively low intensity, so that it is seen only as shoulders or minor peaks to the low-wavelength side of tyrosine and tryptophan bands (Fig. B3.5.10). As a result of the high degree of symmetry in the side chain, the presence of these peaks is an indication of a highly specific and well-packed environment and can act as a marker for native folding.

The ^1L_b transition for **tyrosine** (the ^1L_a transition lies in the far-UV) is more intense than that for phenylalanine and overlaps the two transitions for tryptophan (Strickland, 1974). It can result in a significant contribution to the near-UV CD (e.g., see Fig. B3.5.4 and Fig. B3.5.5) but is not always easy to assign in the presence of tryptophan. Hydrogen bonds to peptide carbonyl, carboxylate (C=O), and imidazole (–N=) groups contribute significantly to its CD, as do the presence of neighboring polar groups. The direction of the optical transition is in the plane of the ring and perpendicular to the axis of symmetry (Fig. B3.5.11) so that interaction with neighboring groups makes the CD very sensitive to rotation of the residue and to the dynamics of the protein in general.

The two transitions for **tryptophan** exhibit distinct features, which lead to quite different CD bands. The ^1L_a transition is broad, relatively featureless, and intense (Fig. B3.5.2). The ^1L_b transition is weaker but exhibits fine structure (Fig. B3.5.2) similar to that of tyrosine. It can be masked by or superimposed on the ^1L_a transition. Interactions similar to those listed for tyrosine strongly affect the peak wavelengths and intensities. The presence of bands above 285 nm is diagnostic for tryptophan in a specific environment; interactions that cause the ^1L_b transition to be shifted to the red (or high-wavelength) end of the spectrum result in bands as high as 310 nm which are highly conformation-specific. The two transitions may be affected quite independently by interactions, and their combination can thereby result in four distinct types of spectrum (Strickland, 1974).

Disulfide bonds may exhibit a broad band of ellipticity in the range of 240 to 350 nm. This can be confused with the band associated with the ^1L_a transition of tryptophan but, in cases where a disulfide makes a significant contribution to the CD, it can be recognized by its ellipticity above 320 nm. It can augment or diminish the apparent intensity of tryptophan and tyrosine contributions without being recognized as such and without fine structure necessarily being lost.

Some of the above features can be seen in the spectra of cathepsin D (Fig. B3.5.10), where the intact single-chain bovine enzyme is compared with the same material cleaved at an exposed loop but without dissociation. The latter has 50% of the specific activity of the intact molecule. Phenylalanine residues can be seen to be present in specific environments in both forms. The fine structure of the ^1L_b transition of tryptophan is superimposed on the broad peak of the ^1L_a transition, which is apparently more intense in the intact enzyme. Alternatively, there could be a greater contribution from disulfide bonds, but the absence of ellipticity above 320 nm favors the former assignment and the CD is therefore consistent with a limited increase in dynamics of the molecule as a result of the chain

cleavage. On the other hand, the persistence of the pronounced peak at 292 nm, where the contribution of the ^1L_a transition is low, indicates that the interaction responsible for this band is unchanged by cleavage. Coupled with the fact that both far-UV CD and fluorescence spectra are identical for the two forms, it is concluded that the overall conformations are structurally similar, differing mainly in their packing and dynamics. Comparison with the spectrum for porcine cathepsin D demonstrates the conformational similarity expected from sequence homology, as evidenced in particular by the persistence of the interaction responsible for the appearance of the ^1L_b band with its intense peak at 292 nm. Differences in the relative intensities of the minor bands provide a sensitive fingerprint for distinguishing two proteins with strong sequence homology.

INTERPRETATION OF FAR-UV CD SPECTRA

SUPPORT PROTOCOL 2

The peptide bond exhibits intense circular dichroism bands when it is located in regular, rigid conformations of the protein backbone chain. It is not surprising therefore to find that the different secondary structures—helix, pleated sheets, and β-turns—each give rise to characteristic spectra (see Fig. B3.5.6). In principle it should be possible, knowing the spectra for each type, to deconvolute the far-UV spectrum for a particular protein to give the proportions of each form of secondary structure in the folded conformation. Early work involved the use of the spectra of polyamino acids as models for α-helix, β-pleated sheet, and random coil conformations. These turned out to be inadequate models for the relatively short helices, distorted intramolecular β-sheets, and aperiodic—but far from statistically random—coil conformations found in the average globular protein. The alternative approach, which forms the basis of most estimations and avoids the need to define reference spectra, involves the use of a database of CD spectra (see Yang et al., 1986, for listings) and corresponding secondary structure contents derived from X-ray structure determination. Linear combination of these spectra is carried out to give a resultant that best fits the experimental spectrum for the test protein. The two algorithms that have been most commonly used are those of Provencher and Glöckner (1981) and of Johnson and coworkers (Johnson, 1990). The former used a bank of 16 proteins with spectra measured between 240 and 190 nm. Using a different statistical approach, Johnson and coworkers provided an algorithm that uses 15 reference spectra measured between 260 and 178 nm. The latter, together with a more recent development of the method, has been reviewed and compared with the Provencher and Glöckner algorithm (Johnson, 1990) and the underlying assumptions have been critically discussed (Manning, 1989). Greenfield (1996) has provided a useful and comprehensive review of all the main algorithms for determining secondary structure. More recent advances in the field are described by Johnson (1999) and Sreerama et al. (1999b). Commercial CD spectrometers include at least one algorithm in their software, although it is worth installing the two original programs independently so that results from each can be compared. Copies are available from the authors of the programs and from Dr. Greenfield.

Attempts have been made to assess the success of different algorithms in deriving secondary structure content from CD spectra (see Yang et al., 1986). The general conclusion is that the helix content is usually estimated well, regardless of the structural type of protein. This is not surprising given the high intensity of the helix spectrum. b-sheet content is estimated well in the case of all-b proteins, but in mixed a and b proteins there is greater uncertainty. Some proteins, such as subtilisin, perform badly regardless of the method used. The estimation of the content of b-turns is similarly subject to greater uncertainty.

Reasons for these problems are various. First, estimations are dependent on the correct values of mean residue ellipticity, which depend critically on determination of the protein concentration. Second, it is reasonably claimed that the shorter the wavelength used to record spectra, the better the estimate of structure (Johnson, 1990). It is not possible with

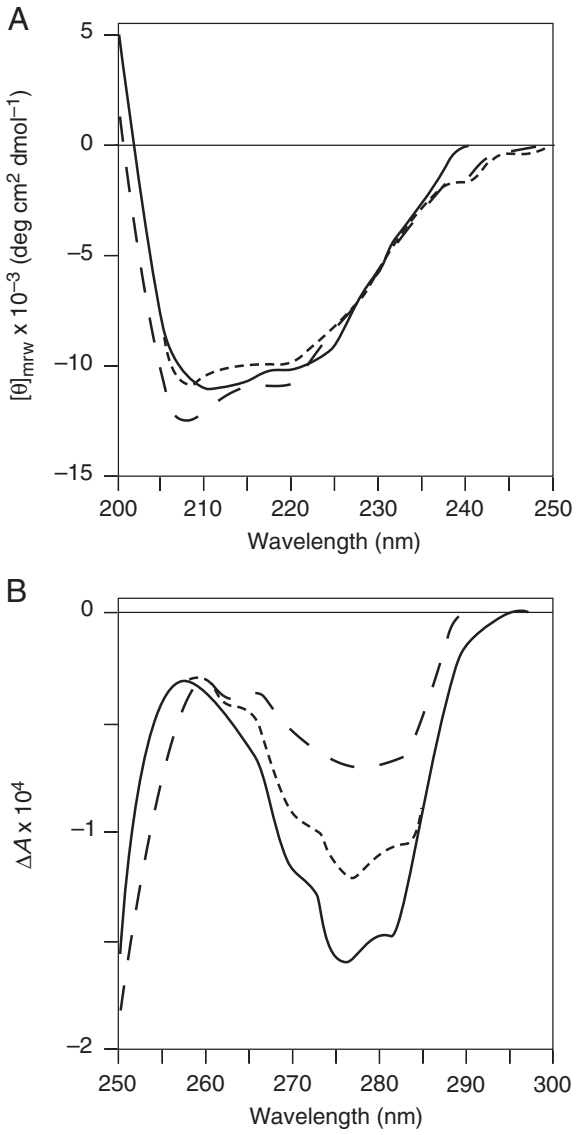

Figure B3.5.12 Effect of mutations detected by CD. The far-UV CD spectra (**A**) show that the secondary structure of β-lactamase PC1 (solid line) from *Staphylococcus aureus* is essentially unaffected by point mutations P2 (Thr 140→Ile; dashed line) and P54 (Asp 146→Asn; dotted line). The crystallographic structure of P54 (Herzberg et al., 1991) confirms that, apart from a loop region, the main body of the molecule that contains the thirteen tyrosine residues is very closely similar to that in the wild-type enzyme. The intensity of the tyrosine ellipticity (**B**) is, however, markedly decreased in each of the mutants, the lower thermodynamic stabilities of which support the interpretation of increased dynamics (Craig et al., 1985).

all proteins to obtain reliable spectra below 185 nm with commercial instruments—inability to use the appropriate buffer and intrinsic scattering from larger proteins are limiting factors, although loss of optimal instrument performance as a result of insufficient maintenance is frequently the main cause of the problem. Third, there is uncertainty about the degree of contribution of aromatic and cystine residues in the far-UV (see Chaffotte et al., 1992, for references and discussion). Aromatic residues are probably significant when the residues are located in clusters, as for tryptophans in the WW domain and for cystine

residues when the dihedral angle is of the order of 90°. Fourth, criteria vary for defining the secondary structure content from the three-dimensional coordinates of structures.

In addition to these problems, the "classical" CD spectrum exhibited by many proteins with a high content of β-sheet secondary structure (Fig. B3.5.6D) is quite different from those for another group of β-structure-containing proteins, of which the first identified was the WW domain (Koepf et al., 1999; not shown), and another is the mushroom inhibitor clitocypin (Fig. B3.5.6C; see Kidric et al., 2002, for further examples). β-casein (Fig. B3.5.6B) shows a similar spectrum, but originating from a more complex mix of conformations (Farrell et al., 2001).

In conclusion, the analysis of spectra properly recorded to 185 nm, or lower where possible, can give useful estimates of secondary structure content, but the content of turns and of β-structure should be interpreted with caution. Fourier transform infrared spectroscopy (FTIR) provides better estimates of the latter. When using the results of far-UV CD determination to characterize reproducibility of folding for different samples, it is important first to compare the spectra visually and to look for possible trends or factors that may explain small differences, rather than to rely solely on comparison of derived secondary structure contents.

COMMENTARY

Background Information

CD spectra in the near-UV region are of much lower intensity than those in the far-UV, in part as a result of the much lower concentration of the chromophore, but can be measured reproducibly with due care. They are very important in characterizing the degree and correctness of folding of recombinant proteins and their mutants.

Near-UV CD depends critically on the atomic environment and closeness of packing of the aromatic residues, including their accessibility to solvent. Although the structural information is limited strictly to the immediate neighborhood of the relatively few aromatic residues, the generally cooperative nature of protein conformation means that changes in aromatic CD frequently reflect more widespread changes in conformation and dynamics. A slight loosening of a protein structure may not result in any change in solvent accessibility to aromatic residues and therefore will not change the maximum fluorescence wavelength of tryptophan. Such a change may also be outside the resolution of X-ray crystallographic analysis. The resulting change in dynamics of the residue, and hence in the asymmetry and detailed interactions with the immediate protein environment, will however affect the near-UV CD spectrum, usually quite markedly (see Fig. B3.5.5 and Fig. B3.5.12).

Changes in the far-UV CD spectrum resulting from slight changes in conformation or partial unfolding will not in general be so marked as in the near-UV region, so that the value of the former for the detailed characterization of correct folding is not as great as that of the latter. It is, however, applicable to proteins with little or no aromatic content. The fact that the far-UV CD arises from the transitions of a single type of chromophore—i.e., the peptide bond—and that the backbone conformation constitutes the main influence on its dichroism, means that this region is good for identifying family relationships between proteins.

A number of examples in the literature illustrate the varied contributions of CD in examining food proteins and their reactions. For instance, γ-radiation is shown to disrupt the conformation of ovalbumin, but the protein's conformation can be protected by the presence of ascorbic acid (Moon and Song, 2001). New structural and stability properties were shown for β-lactoglobulin isolated by a mild procedure (de Jongh et al., 2001). Structural changes induced by heating gluten vary with moisture content (Weegels et al., 1994). The setting of fish actomyosin is proposed to require unfolding of α-helical structure (Ogawa et al., 1995). The effects of high pressure on β-lactoglobulin, ovalbumin, and lysozyme were studied by Tedford et al. (1999a,b), and the reversibility of heat-induced heme dissociation from horseradish peroxidase was determined by Adams et al. (1996).

CD in general is a useful tool for examining proteins made by site-directed mutagenesis for structure-function studies, where it is important to know to what extent the conformation has been

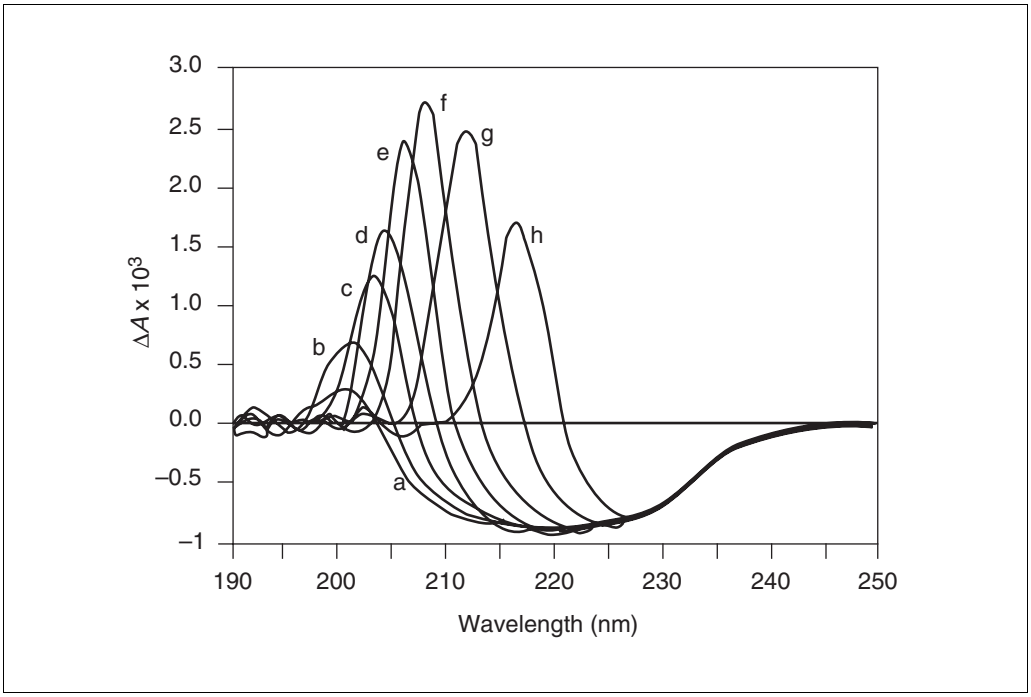

Figure B3.5.13 A convincing artifact. In an attempt to study the conformational consequences of adding an acceptor, D-glutamine, to a DD-peptidase, far-UV CD spectra were recorded for the enzyme in the presence of increasing concentrations of glutamine: a = 0 mM, b = 3 mM , c = 7 mM, d = 10 mM, e = 20 mM, f = 30 mM, g = 50 mM, and h = 95 mM. The enzyme concentration was 0.1 mg/ml, equivalent to ~10^{-3} M peptide bond, in 10 mM sodium phosphate pH 7.2. A 2-mm cell path length was used.

affected by the mutation. The far-UV spectrum will tell whether the mutant has folded with a backbone conformation similar to that of the wild-type protein, whereas the near-UV spectrum will detect many minor changes in conformation or dynamics (Fig. B3.5.12).

CD complements fluorescence as a technique for characterizing the folded conformation in solution and for providing, in a single spectrum, a highly specific fingerprint for the native state. It requires only modest amounts of protein and measurements are relatively quick to make. Like fluorescence, its use as a comparative method requires a control spectrum from a carefully characterized sample of the natural or recombinant protein, recorded under defined and optimal conditions, using a spectrometer in good condition.

Critical Parameters

Many of the critical experimental parameters for CD spectrometry are the same as those for measuring fluorescence. Spectra will only be as reliable as the protein solution, the machine, and the operator. Cells must be scrupulously clean, solutions must be well-clarified, and buffers must have a low absorbance. In addition, the protein concentration must be known precisely and, together with the path length, must be chosen to optimize the absorbance in the region of interest. This will sometimes require recording a spectrum in overlapping sections. Preliminary experiments to optimize the instrumental parameters are important. Lastly, it is essential that the spectrometer be well maintained and up to specification. The useful life for a lamp is on the order of 800 to 1000 hr, but performance in the far-UV may start to fall off before this.

Troubleshooting

The problem most frequently met is the loss of light intensity at low wavelengths (though a casual scan of the literature shows that it is not so frequently recognized). Under such conditions, many spectrometers will produce traces that are smooth, giving the appearance of being valid spectra, but which are in fact artifacts. The example of such an artifact shown in Figure B3.5.13 provides a cautionary tale. In this case, the glutamine absorbs light in the far-UV on account of its side-chain amide group. The increasing concentration of glutamine results in a progressively longer wavelength at which light is cut off, to which this particular spectrometer responded by returning to zero di-

chroism, giving the impression of a peak with a maximum at increasing wavelengths. To avoid such artifacts, it is essential to monitor the photomultiplier high voltage during a scan. Similar problems can arise from using too high a protein concentration or too long a light path, from too much absorbance by the buffer, from turbidity in the protein solution, from a dirty cell, or—below 200 nm—from absorption by oxygen that results from incomplete purging with nitrogen.

A further problem that is sometimes encountered is that the spectrum obtained after baseline subtraction does not return to zero above 320 nm (in near-UV CD) or 250 nm (in far-UV CD). If, above these wavelengths, the spectrum runs parallel to the zero base, a provisional spectrum may be derived by subtracting the difference in ellipticity, as a constant, from the whole spectrum. For quantitative assessment of a sample, however, the spectrum should be redetermined, paying attention to the correct buffer blank, cell reproducibility, and instrumental drift (see Strategic Planning).

Care must be taken to rectify all of the above mentioned problems if meaningful spectra are to be obtained for reliable characterization.

Anticipated Results

Several examples of near- and far-UV CD spectra are given in the figures included in this unit. With current instruments the final spectra exhibit low noise and—provided that the instrument and sample parameters have been optimized—should be true and reproducible within the limits of protein-in-buffer absorbance of <1. The most frequent source of nonreproducibility of intensity in spectra is the difficulty of determining the protein concentration, particularly of larger proteins that scatter more and of those with a greater tendency to aggregate.

What constitutes a significant difference between two spectra? When the differences are small, the answer depends on sample preparation and sample stability as well as accuracy of concentration determination, identification of and compensation for drift in the spectrometer, correct baseline correction, absence of bubbles in the sample, reproducible cleanliness of the cuvette, and the level of general handling procedures. Ultimately, an assessment of significance depends on the experience, competence, and confidence of the operator.

As CD is very sensitive to small changes in conformation and dynamics, wild-type and mutant proteins will frequently give spectra that differ slightly in intensity rather than shape. If the dihedral angle for a disulfide bond is such as to yield an intense contribution, small conformational perturbations in a mutant protein may further affect the spectrum. Hence, there is the need to examine proteins by a number of different spectroscopic, hydrodynamic, and functional probes in order to assess correct folding of mutant proteins.

Time Considerations

A single spectrum, with associated baseline, will require ~1 hr, including the time needed to fill and clean the cuvette. Up to an additional hour may be needed for optimizing instrument parameters if the sample is unfamiliar. For a number of samples, economies of time can be effected (see Basic Protocol).

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Determining the Fluorescence Spectrum of a Protein

Fluorescence spectra provide a sensitive means of characterizing proteins and their conformations. The uses of fluorescence are limited only by the need for a chromophore that can absorb ultraviolet or visible light and re-emit it at a longer wavelength as fluorescence. The characteristics of the second of these two processes depend sensitively on the physical environment of the chromophore or on the nature of any chemical group to which it may be covalently attached. Thus, the tyrosine or tryptophan residues in a protein will fluoresce in a manner critically dependent on the folding of the protein, and act as sensitive monitors of conformational change in response to denaturation by heat, pressure, high or low pH, and so on. Similarly, a dye or other molecule such as retinol with a conjugated electronic structure will fluoresce differently when its environment is changed by noncovalent binding to another molecule, surface, or micelle. If a fluorescing molecule such as dinitrophenol is covalently attached to another molecule such as a peptide, cleaving the attaching bond or one close to it will induce a change in fluorescence.

It can immediately be seen that monitoring any of these changes in fluorescence can form the basis of an assay for following conformational change in macromolecules, determining the polarity of micelle surfaces, or assaying a variety of lytic enzymes, all of which are directly applicable to practical problems of food texture and stability. Since fluorescence can be monitored by relatively simple detectors that can respond rapidly to changes in light intensity, it can be used to follow the kinetics of processes occurring over periods of minutes to nanoseconds. In fact, its main use is as a monitor of change; detailed knowledge of the nature of the change requires understanding of the agents bringing about the change, coupled with results of other methods such as circular dichroism, NMR, hydrodynamic, and scattering techniques.

One of the main values of fluorescence as a technique for probing protein conformation is that it is highly sensitive and very economical of material. This means, however, that small traces of fluorescent impurities in the solvent or on the cell are readily detected and, if care is not taken, can lead to misinterpretation of the spectra. The essential aim in using this technique, therefore, must be to obtain a fluorescence emission spectrum for a protein that is guaranteed free from all too easily generated artifacts.

STRATEGIC PLANNING

The following points are essential to obtaining reproducible spectra for reliable comparison between samples.

Fluorescence Spectrometer

Fluorescence spectra are measured with a fluorescence spectrometer (spectrofluorometer), of which there are a variety of models on the market. For protein characterization, a xenon light source and optics that ensure low levels of stray light are essential. Spectrometers with dual holographic gratings in the excitation monochromator are preferred. There are two main types of fluorescence detection available: analog and photon counting. The latter counts individual photons, which enables high sensitivity and stability, but limits the range of intensities over which a linear response is obtained. Analog spectrometers provide high-precision measurements over a wide range of intensities. Stability is generally high, but is improved by the presence of a reference photomultiplier. In general, the photon-counting spectrometer is for more specialized use and the analog type of instrument is more widely used for the kind of work described in this unit.

Contributed by Roger H. Pain
Current Protocols in Food Analytical Chemistry (2003) B3.6.1-B3.6.21

A thermostatted cell holder—either one connected to a good circulating thermostat bath or, preferably, one of the Peltier controlled type—is essential. The latter controls the temperature by electrical means, the advantages being that no water needs to be brought into the spectrometer and that the temperature can be both raised and lowered very quickly. The spectrometer should incorporate shutters that protect the sample and the photomultiplier from radiation damage when scanning is not in progress. A magnetic stirrer incorporated into the cell holder is useful to facilitate mixing and to minimize artifacts due to photochemical degradation in the small volume of the excitation beam. Careful attention must be paid to the manufacturer's instructions in order to achieve the optimum settings for measurement.

Calibration of Spectrometer

In order to ensure reproducibility, the wavelength setting should be correct within ±0.5 nm. This will generally not be a problem, but it is just as well to check, most readily with a mercury lamp inserted into the cell compartment. The procedure described by Lakowicz (1983) is recommended. Calibration of intensity can be more difficult, and it is always more reliable to scan samples in parallel with a solution of a known, stable compound, be it the protein in question or a model compound such as *N*-acetyl-L-tryptophanamide.

Cells

Choice of cells

The fluorescence cuvettes best used for reliable characterization of proteins are either the standard 10 × 10–mm fluorescence quartz cuvettes (with all four faces polished), taking 2 to 3 ml of solution, or semimicro cuvettes, taking ~0.7 ml. In order to ensure reproducible positioning of the cuvette, it should be placed in the cell holder with the inscription facing the incident light beam.

Preparation and handling of cells

Cuvettes must be thoroughly and reproducibly cleaned by soaking in a proprietary cuvette-cleaning fluid such as Hellmanex II (Hellma), an alkaline liquid concentrate, or RBS-35 Detergent Concentrate (Pierce), following the manufacturer's instructions. The manufacturers of Hellma cuvettes caution against the use of ultrasonics as a means of boosting cleaning efficiency. Fifty percent nitric acid is also effective, but requires greater care and a fume hood. Detergents frequently contain fluorescent material and, if used, should be removed from cuvettes with 2 M HCl followed by water. After the final rinse with copious amounts of high-quality distilled water, the cells are best dried using suction through a Pasteur pipet protected with a small length of plastic tubing. This is safer than using acetone or alcohol, which can leach out fats from fingers and deposit them on the cuvette. The faces of cuvettes must not be touched after cleaning. Filling, emptying, and rinsing between measurements of spectra must be done using pipets, to avoid spillage onto the outer faces. A Pasteur pipet protected with fine plastic tubing is satisfactory. The baseline scans, with buffer in the cell, must be examined as a constant check on the reproducibility of the state of the cell.

The cleanliness of the cuvette can be checked by filling with distilled water and scanning the appropriate wavelength region of the spectrum. If there is no contamination by fluorescent material, the spectrum should be flat, save for the Raman band (see Fig. B3.6.1) on the high-wavelength side of the excitation wavelength, λ_{max}.

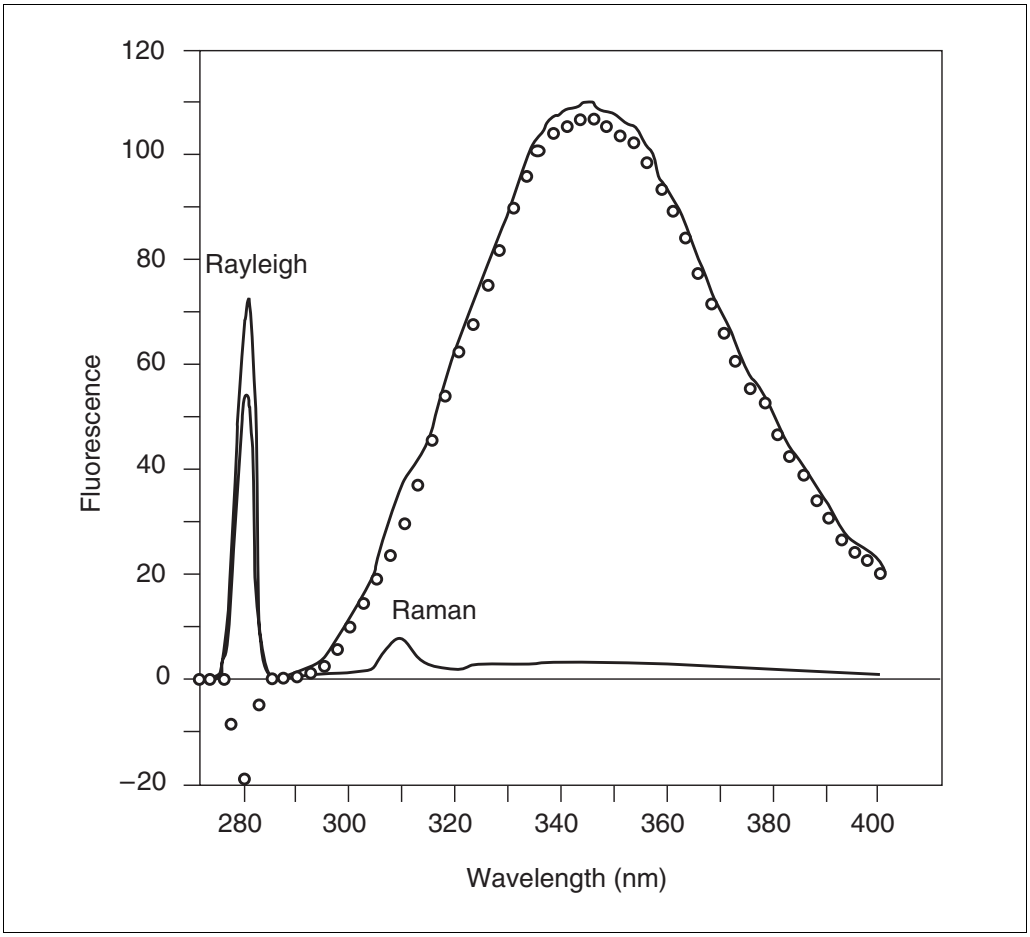

Figure B3.6.1 Rayleigh and Raman bands in fluorescent spectra, as seen in scans for solvent baseline and hen egg white lysozyme (EWL) solutions (solid lines). Circles represent the spectrum of EWL with baseline subtracted. Parameters: EWL A_{280} = 0.05; λ_{ex} = 280 nm; excitation and emission bandwidths, 2.5 nm; scan rate, 100 nm/min; five scans accumulated. Spectra were measured using a Perkin Elmer LS50B fluorescence spectrometer.

Buffer Solutions

Buffers should have low absorbance and low fluorescence in the regions of excitation and emission. Absorbance will usually be expected to be <0.1 and fluorescence close to zero. This will normally be the case for standard buffers made from analytical-grade reagents or materials of equivalent purity, but they should nevertheless be checked routinely for fluorescence. If a fluorescent component is added to the solution—e.g., as a ligand—it should be checked that the observed fluorescence arises solely from that component. The actual buffer solution used to dissolve or to dialyze the protein should be used for the fluorescence blank. Plastic containers (and stirring bars) may contribute fluorescent agents; if these are used, appropriate blanks should be carefully monitored for fluorescence.

Clarification of Solutions

Scattering of light from particles in the light beam will be seen as fluorescence, and must therefore be rigorously avoided. Protein solutions must be freed from the turbidity resulting from dust and aggregated protein. Many proteins can be filtered (preferably as a stock solution) using a 0.22-μm filter—ideally of the grade special for proteins such as Durapore (Millipore)—fitted to a syringe. Alternatively, if the protein is adsorbed by filters, the solution can be clarified by centrifugation. A microcentrifuge operated at maximum speed for 10 min at 4°C provides reliable clarification for the majority of

proteins that do not sediment appreciably under these conditions. Buffer solutions are filtered on a larger scale using a 0.22-μm filter.

It is essential to monitor clarification if artifacts are to be avoided. There are three indicators of the completeness of clarification, each available from the standard procedures that should be adopted for spectroscopic characterization.

1. Determination of protein concentration (*UNIT B1.3*) requires an absorbance spectrum to be recorded on a good quality spectrophotometer from 240 to 350 nm. Aromatic amino acid residues do not absorb above 320 nm, so the spectrum between 320 and 350 nm should be only marginally above baseline. The presence of turbidity will result in finite attenuance in this region that increases toward lower wavelengths.
2. From the above absorbance spectrum, the ratio A_{max}/A_{min} is measured, where A_{min} is the absorbance at the trough in the region 240 to 250 nm. This ratio will be specific for a given protein and should be measured for a sample that has been carefully and reproducibly clarified. Due to the strong wavelength dependence of scattering, the ratio is very sensitive and decreases sharply with turbidity. It can be used as a sensitive check on the clarity of further solutions of the protein.
3. The scan of a fluorescence emission spectrum should routinely include the region of the Rayleigh peak (see Fig. B3.6.1). This provides a built-in indicator of turbidity of the buffer and protein solutions. The height of the peak for the protein solution should not differ greatly from that obtained for the solvent from the equivalent buffer blank scan; it will increase markedly if there is dust or aggregate in the solution.

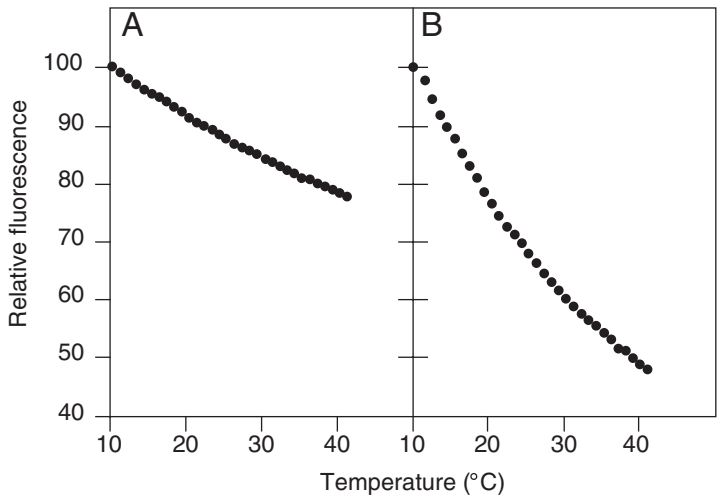

Figure B3.6.2 Temperature dependence of fluorescence in a polar environment. (**A**) Tyrosine (6 μM) at 303 nm (λ_{ex} = 274 nm). (**B**) Tryptophan (1 μM) at 355 nm (λ_{ex} = 278 nm). Both chromophores are in a polar environment, 0.01 M potassium phosphate, pH 7.0. The dependence is much smaller in a nonpolar environment. Reprinted from Schmid (1989) with permission of Oxford University Press.

RECORDING A FLUORESCENCE EMISSION SPECTRUM

A fluorescence emission spectrum is the plot of fluorescence intensity as a function of wavelength. Fluorescence emission takes place from the constant wavelength of excitation (λ_{ex}) upwards. The range of the spectrum recorded is described below (step 5).

Materials

Calibrated fluorescence spectrometer, buffer solution, and cleaned cuvettes (see Strategic Planning)

Clarified protein solution of known concentration (see Strategic Planning for clarification methods and *UNIT B1.3* for concentration determination) with absorbance usually in the range of 0.05 to 0.1 at the wavelength to be used for excitation

Prepare spectrometer

1. Switch on the spectrometer and allow it to warm up for the time specified in the manufacturer's instructions (usually ~30 min). Regulate the thermostat control to the desired temperature (normally 25°C unless the stability of the protein dictates otherwise).

   The intensity of fluorescence is highly temperature-dependent (see, e.g., Fig. B3.6.2), so it is important that the temperature be accurately controlled and measured in the cuvette with a calibrated electric thermometer. It is useful for future reference to note the time taken for the sample to reach the operating value.

2. Set the excitation wavelength (λ_{ex}).

   Setting λ_{ex} to the λ_{max} of the absorbance spectrum (usually 280 nm) will excite both tyrosine and tryptophan residues. Alternatively, λ_{ex} may be set to 295 nm in order to excite only tryptophan (see annotation to step 3).

3. Set the excitation bandwidth.

   The term bandwidth is described and defined in UNIT B3.5. The excitation bandwidth (in nm) is determined by the excitation slit width (in mm). It will determine the intensity of the excitation and the amount of scattered light from the Rayleigh peak that will be "seen" in the emission spectrum. It is important in certain cases in determining which chromophore is excited. It is particularly important, for example, to limit the bandwidth when measuring the contribution of tyrosine in the presence of tryptophan. The emission maximum of tyrosine at 303 nm (see Fig. B3.6.3) is close to the required excitation wavelength of 295 nm. The envelope of the Rayleigh peak must not, in this case, significantly overlap the emission band of tyrosine. Settings of 2.5 to 5 nm for $\lambda_{ex} \approx 280$ nm and ≤ 2.5 nm for $\lambda_{ex} \approx 295$ nm are usual.

4. Set the emission bandwidth.

   Since protein emission spectra are generally rather broad, larger emission bandwidths can usually be tolerated. Only where it is important to resolve tyrosine fluorescence from tryptophan fluorescence and the Rayleigh scattering peak is it necessary to minimize the bandwidth. In cases where only low concentrations of material are available, it is necessary to strike a balance between resolution and light intensity in order to obtain the best possible signal-to-noise ratio. Settings of 2.5 to 10 nm are normal.

5. Set the range of emission wavelengths to be scanned for the emission spectrum.

   The lower wavelength should be ~10 nm below the excitation wavelength in order to record the Rayleigh peak (see Strategic Planning, discussion of Clarification of Solutions). The higher end of the range should be chosen to include as much of the spectrum as possible, so as to allow integration of the peak if required. Values of 400 and 340 nm are suitable for proteins with and without tryptophan, respectively. For example, a typical emission wavelength range for a Trp-containing protein excited at 295 nm would be 285 to 400 nm.

6. Set the scan rate.

The scanning rate for the emission wavelength has to be slow enough to allow the instrument to respond to changes in intensity with wavelength. Too fast a scan, even with a number of accumulations, results in an apparent spectrum that can differ markedly from the true spectrum (Fig. B3.6.4). When, as is usually the case with proteins, the emission band is rather broad (Fig. B3.6.1), a scan rate setting in the region of 100 nm/min will usually be appropriate.

Scan buffer blank and protein sample

7. Fill a cleaned cuvette with a filtered sample from the actual batch of buffer used to dissolve or dialyze the protein, then place the cuvette in the cell holder of the spectrometer in a reproducible orientation (see Strategic Planning, discussion of Cells). Scan this buffer blank using the same instrument settings as are appropriate for the sample, store the spectrum, and check for any unexpected fluorescence bands.

If the settings appropriate for the protein sample cannot be estimated in advance, the sample may be run before the buffer blank. Special care will have to be taken to remove any protein adsorbed to the inner faces of the cuvette.

Temperature equilibration is not usually important for the buffer, except in cases where it contains fluorescent components.

Cell holders are susceptible to corrosion by salt solutions. Cells should therefore be filled outside the spectrometer. Special care must be taken to position the cuvette reproducibly each time.

Apart from the Raman band, a buffer solution should give an almost flat baseline of low intensity (see Fig. B3.6.1). At the usual working concentrations of protein (A_{280} ≥0.05), buffer fluorescence constitutes only a minor contribution to the fluorescence spectrum of the protein and is accounted for by the appropriate baseline subtraction.

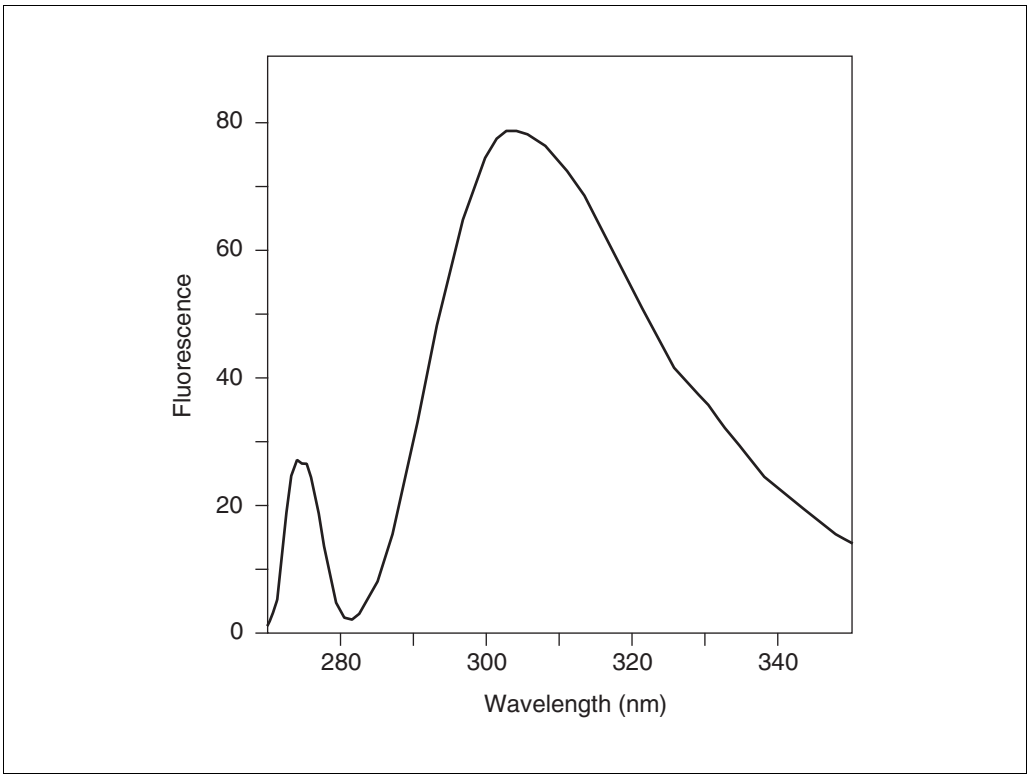

Figure B3.6.3 Fluorescence spectrum of a "tyrosine only" protein, ribonuclease (RNase). Parameters: RNase A_{275} = 0.05; λ_{ex} = 275 nm; one scan, smoothed; other settings as for Figure B3.6.1. Baseline was measured with the same settings and subtracted. Measurements made using a Perkin-Elmer LS50B.

It is advisable to repeat baseline scans at intervals during a series of experiments to check on contamination of the outer faces of the cuvette, and of the inner faces due to protein adsorption.

8. Remove the cuvette from the spectrometer, empty with a protected Pasteur pipet (see Strategic Planning, discussion of Cells) and refill in the same way with the clarified protein solution of known concentration with an absorbance of 0.05 to 0.1 at the wavelength used for excitation. Allow to come to temperature in the cell holder, then scan and store spectrum.

If the protein concentration has to be low, and/or the intensity of fluorescence is low, it may be necessary to accumulate a larger number of scans. Proteins differ widely in their sensitivity to photodegradation, but exposure to UV radiation may lead to damage to those molecules in the light path, with subsequent reduction in fluorescence intensity. Excitation slit widths should, therefore, be as narrow as is consistent with good signal-to-noise ratio. Stirring the solution in the cuvette will bring undamaged molecules into the light path (Fig. B3.6.5) and further reduce significant artifacts due to radiation damage.

Calculate results

9. Subtract the baseline from the sample spectrum using the instrument software (Fig. B3.6.1).

Fluorescence has no units. Hence, if the fluorescence intensities of different samples are to be compared, careful attention must be paid to concentrations and instrumental conditions. Instrument response may vary with time, and thus must be controlled by the use of stable standards.

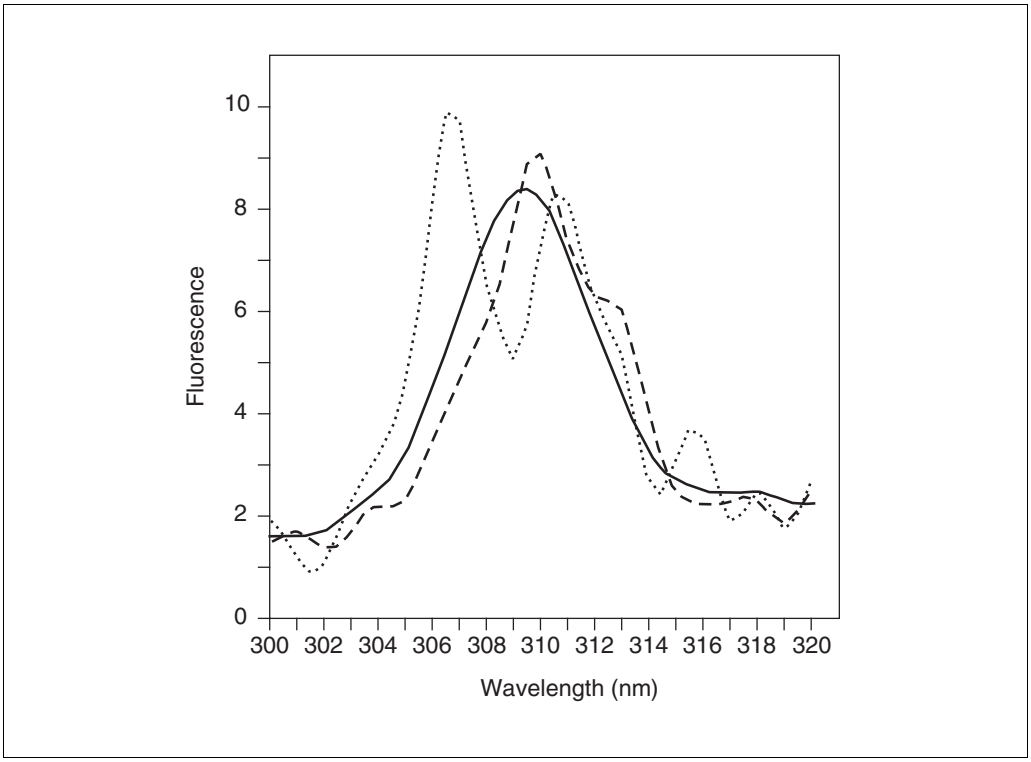

Figure B3.6.4 The effect of scanning parameters on the authenticity of spectra. The Raman band from Figure B3.6.1 is shown for a single scan at 1500 nm/min (dotted line); ten scans at 1500 nm/min (dashed line); and ten scans at 100 nm/min (solid line). The times required for scanning the complete baselines for these three measurements were 0.087 min, 0.87 min, and 13 min, respectively. Measurements made using a Perkin-Elmer LS50B.

10. Correct the apparent fluorescence intensities for the inner filter effect.

A protein solution will absorb a certain proportion of the radiation before it reaches the volume of solution whose fluorescence is viewed by the optics of the instrument (see Fig. B3.6.5). For a solution of absorbance 0.1, the intensity at the center of the cell is about 10% less than that of the incident radiation, with a corresponding decrease in the observed fluorescence. Similarly, the fluorescence intensity may be reduced by absorption in the exit path. This is known as the inner filter effect. In practice, it means that the observed fluorescence intensity is directly proportional to protein concentration only at low concentrations, when absorbance is <0.1 at λ_{ex}. The apparent fluorescence intensity (F_{app}) can be corrected by the following approximation:

$$F_{corr} = F_{app}\, antilog\{(A_{ex} + A_{em})/2\}$$

where A_{ex} and A_{em} are the solution absorbances at the excitation and emission wavelengths, respectively.

The effect is much more marked if a third component that absorbs in the wavelength range of the protein absorption or fluorescence is added to the solution (see Basic Protocol 2).

11. Determine the value of the fluorescence intensity at the wavelength of maximum intensity (I_{max} at λ_{max}) using, where available, the instrument software.

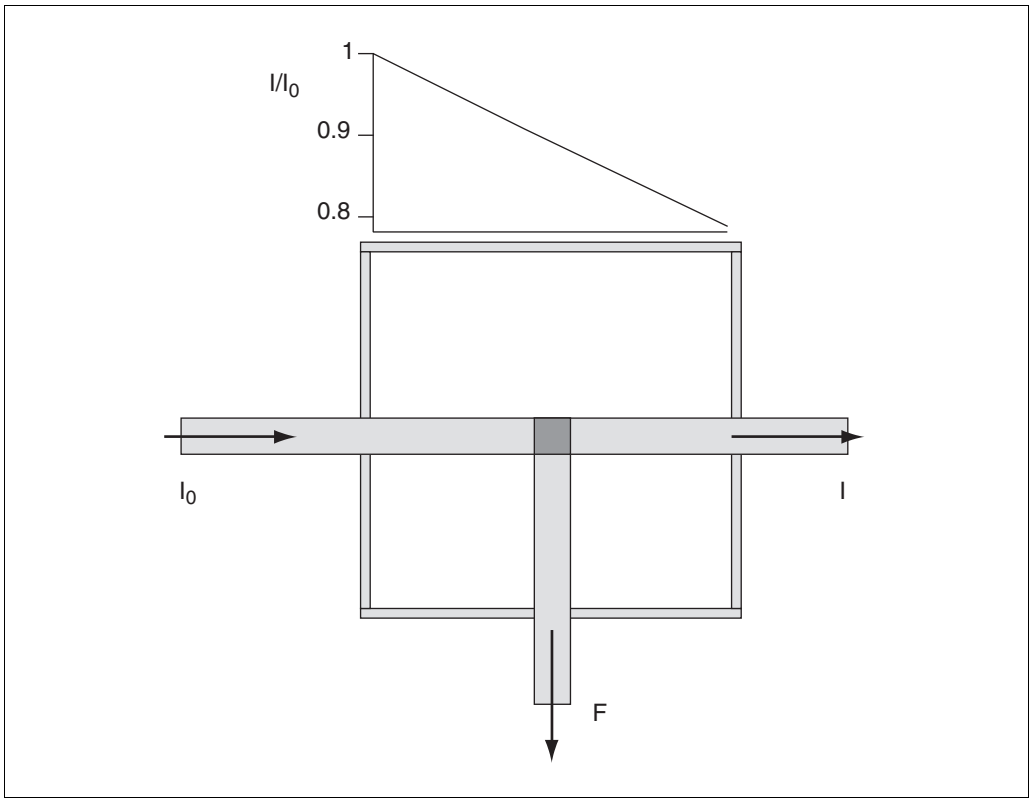

Figure B3.6.5 The inner filter effect. A cuvette (10 × 10–mm) is represented in plan view, with the collimated incident beam from the monochromator having intensity I_0. As a result of absorption by the protein solution, the intensity of the beam through the cuvette will decrease steadily, emerging with intensity *I*. The values are illustrated for a solution having an absorbance at the excitation wavelength of 0.1. The optics of the fluorescence detector are focused so that only fluorescence originating from the volume depicted by the heavily shaded square is "seen" by the photomultiplier. Thus the observed normalized fluorescence intensity will be less than that expected from the protein at infinite dilution. The fluorescence passes through the protein solution on its way to the detector and will be further decreased in intensity if the solution absorbs at the wavelengths of the emitted radiation.

It is possible to obtain corrected spectra, which take into account the variation of intensity of the light source as well as variation of the detector response with wavelength. The procedures for calculating these are not simple (see Lakowicz, 1983), and for the purposes of the characterization described here the uncorrected spectra are normally used. Because of the above variations, however, it is important when comparing spectra to do so with spectra obtained using the same instrumental settings and, preferably, the same instrument.

In order to compare complete spectra from different samples of a protein, they should be normalized by dividing the value of fluorescence intensity by the protein concentration (i.e., I_{em}/c).

DETERMINATION OF FLUORESCENCE QUENCHING

BASIC PROTOCOL 2

Fluorescence quenching provides a means of probing the accessibility of tryptophan residues to small molecules and thus gives information about their structural environment. The technique involves quantifying the decrease in protein fluorescence intensity in the presence of increasing concentrations of quencher, followed by analysis of the data to give details of the interaction of the quencher with the tryptophan residue.

Materials

Protein solution in buffer, A_{280} = 0.05 to 0.1

Quenchers: these are most conveniently made up as concentrated stock solutions; examples of frequently used quenchers include:

5 M NaI or 2.5 M KI solution containing 1 mM $Na_2S_2O_3$ to prevent I_3^- formation

5 M CsCl (optical grade, Aldrich)

8 M acrylamide (Electran grade from BDH; ε_{295} = 0.236 liter/mol/cm)

2.5 M succinimide (recrystallized from ethanol with activated charcoal treatment; ε_{295} = 0.03 liter/mol/cm)

Additional reagents and equipment for recording a fluorescence spectrum (see Basic Protocol 1)

1. Scan the fluorescence spectrum of a measured volume of the protein in the required buffer (see Basic Protocol 1).

2. Add a known small volume of a stock solution of quencher, mix thoroughly and again scan the spectrum.

   Alternatively, for economy of time, measure the intensity of fluorescence at just two wavelengths—315 and 350 nm—allowing a sufficient time of accumulation to achieve reproducible average readings at each wavelength.

   The actual amount of quencher added is a matter of judgment, depending on the degree of quenching found. It is important to obtain at least ten measurements of fluorescence intensity spread evenly between zero and full quenching (see Fig. B3.6.6).

   Quenchers should be carefully purified. When iodide is used, it should be in the presence of 1 mM $Na_2S_2O_3$ to avoid the formation of I_3^-, which can react with tyrosine.

   The tryptophan residues in a given protein are likely to be in different environments and, therefore, to exhibit different values of λ_{max}. Measurement of the degrees of quenching at the red and blue ends of the emission spectrum will determine whether this is so and will also add further detail to the overall fingerprint of the protein (see Support Protocol, discussion of Quenching).

3. Correct each reading of the fluorescence intensity for dilution and for the absorbance of the quencher (see Basic Protocol 1).

   See also Eftink and Ghiron (1981).

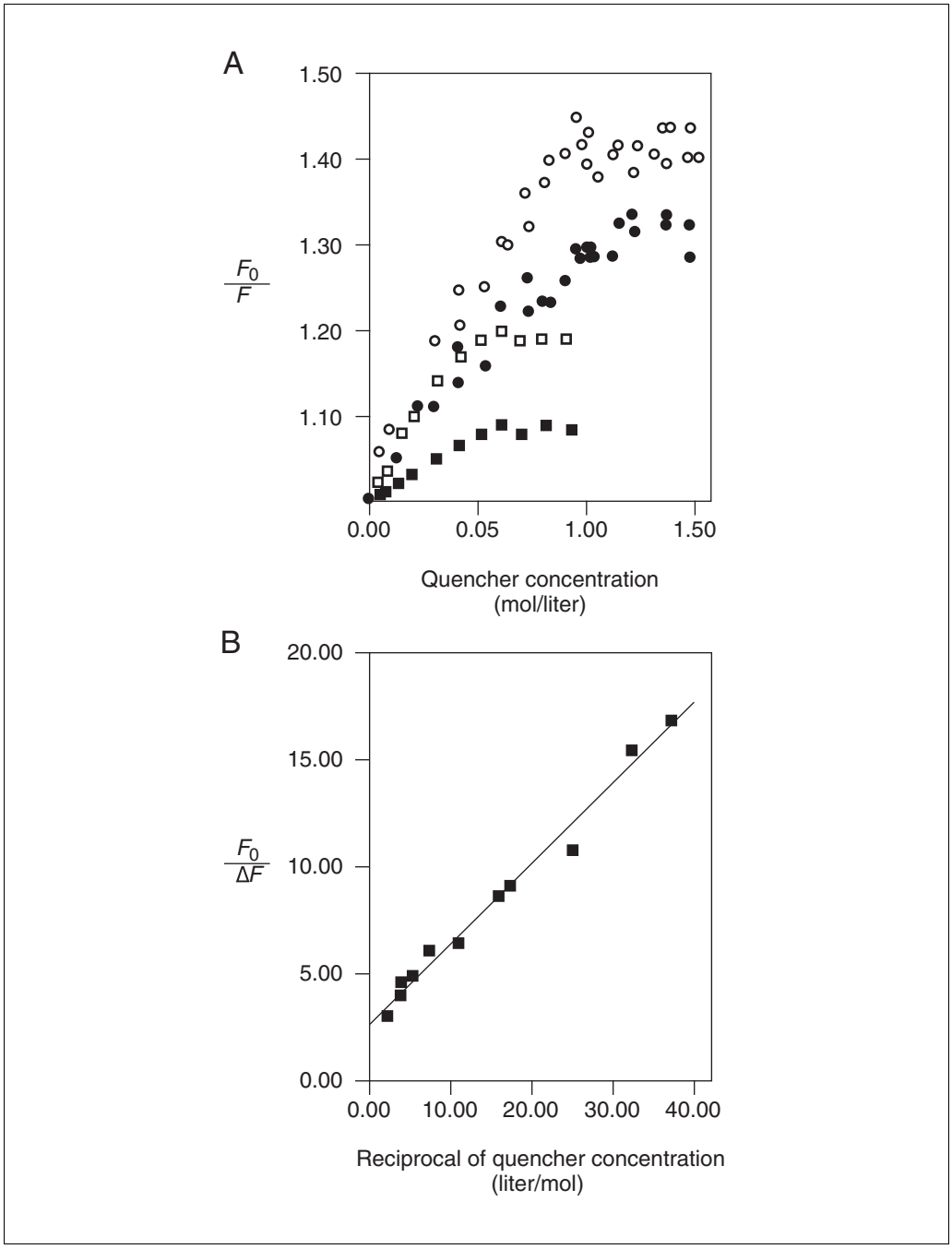

Figure B3.6.6 Fluorescence quenching by different quenchers–analysis of data. (**A**) Fluorescence intensities from 3-phosphoglycerate kinase (PGK), expressed as a fraction of the unquenched fluorescence, are plotted according to the Stern-Volmer equation (see Basic Protocol 2, step 4) at 316 nm (filled symbols) and 350 nm (open symbols). Quenchers used were succinimide (circles; treated with activated charcoal and recrystallized with ethanol; 2.5 M stock solution) and potassium iodide (squares; 2.5 M stock solution with 1 mM $Na_2S_2O_3$). Fluorescence intensities were adjusted for dilution and for quencher absorbance using a molar extinction coefficient ε_{295} = 0.03 liter/mol/cm for succinimide. The higher level of quenching at 350 nm reflects the fact that the more exposed of the two tryptophan residues has a more red-shifted fluorescence maximum. (**B**) Modified Stern-Volmer plot for the quenching of PGK fluorescence by succinimide. The data in panel A for quenching at 350 nm is plotted according to the modified Stern-Volmer equation (see Basic Protocol 2, step 4 annotation). The intercept on the *y*-axis is 2.36 ± 0.2. The results in panels A and B are characteristic of a protein where part of the fluorescence is emitted by residues(s) that are inaccessible to a given quencher, in this case, iodide and succinimide. The tryptophan(s) are less accessible to iodide than to succinimide, with implications for the polarity of their environment. Measurements made using a Perkin-Elmer MPF3-L. Reprinted from Varley et al. (1991) with permission of Elsevier Science.

4. Plot the resulting fluorescence intensities as a function of concentration of quencher according to the Stern-Volmer equation (see Fig. B3.6.6A):

$$F_0/F = 1 + K_D[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence, respectively, of a given concentration of quencher, [Q], and K_D is the Stern-Volmer quenching constant.

K_D is determined by the bimolecular quenching constant (k_q) and by the fluorescence lifetime of the fluorophore (τ_0), in the absence of quencher ($K_D = k_q\tau_0$). The slope of a plot of F_0/F against [Q] gives K_D directly, in units of liters/mol, with an intercept of unity. This plot is expected to be linear for a single fluorophore in a single environment. For a more complex system the plot will be non-linear (see Fig. B3.6.6A). Assuming that there are two populations of trytophans—one (a) accessible to quencher and the other (b) buried and inaccessible—the data can be plotted according to a modified Stern-Volmer equation:

$$F_0/\Delta F = 1/f_a K_D[Q] + 1/f_a$$

where f_a is the fraction of the initial fluorescence accessible to the quencher

$$f_a = F_{0a}/(F_{0a} + F_{0b})$$

and K_D is the Stern-Volmer quenching constant for that fraction of the fluorescence. A plot of $F_0/\Delta F$ against $1/[Q]$ gives an intercept of $1/f_a$ and a slope of $1/f_aK_D$ (see Fig. B3.6.6B).

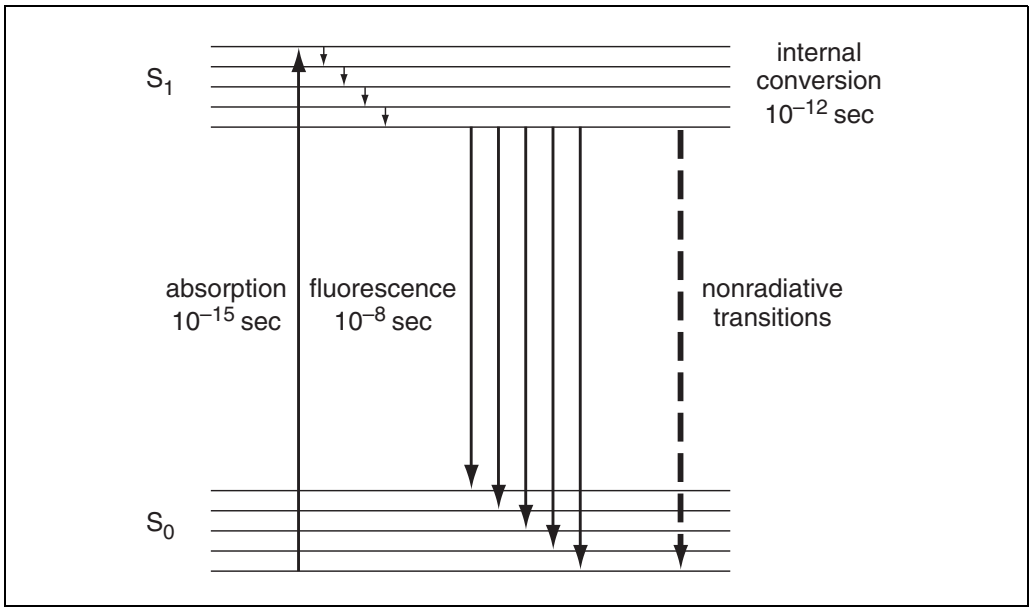

Figure B3.6.7 The processes of absorption and emission of radiation by a chromophore. The electronic structure of a chromophore is in the ground state (S_0) under normal conditions. Absorption of radiation, a rapid process, leads to excitation to the vibrational states of an excited state (S_1). Under normal thermal conditions, the excitation energy is rapidly degraded to the lower one or two vibrational levels. Thus, although excitation takes place into a range of levels in the excited electronic state, emission, in the form of fluorescence, takes place from the lowest vibrational level to a range of vibrational levels in the ground electronic state. The lengths of the vertical lines representing absorption and emission illustrate the relative energies of the radiation in each process and illustrate why fluorescence almost always occurs at longer wavelengths than absorption. The vertical broken line represents the dissipation of absorbed energy by means other than radiation.

BASIC THEORY AND INTERPRETATION OF FLUORESCENCE SPECTRA

When radiation is incident on a solution, most of it is scattered without change in wavelength. Thus, exciting radiation at 280 nm will be scattered and a fraction of it will be detected by the spectrofluorometer as a peak at the same wavelength. The width of this *Rayleigh* scattering peak (Fig. B3.6.1) will depend on instrumental settings, mainly those of the excitation and emission slit widths, which determine the range of wavelengths observed. The *bandwidth* is the width, in nm, of the distribution of intensity measured at half the height of the maximum intensity.

A further low-intensity peak, known as the *Raman band*, is also observed in spectra (see Fig. B3.6.1). This is a low-intensity band of scattered radiation whose distance from the excitation band is a measure of the vibrational energy of the H–O bond in solvent water. At λ_{ex} = 280 nm, the Raman band for water occurs at 311 nm; in general, it occurs at $1/\{(1/\lambda_{ex}) - (3.6 \times 10^{-4})\}$, where λ_{ex} is the excitation wavelength in nm. The intensity and resolution of the Raman band provide a useful empirical check on the performance of the spectrofluorometer. A decrease in the signal-to-noise ratio usually indicates a deterioration of the lamp.

Chromophores, such as the sidechains of tryptophan and tyrosine, will absorb some of the radiation in ~10^{-15} sec and become excited from the ground electronic state (S_0) to higher vibrational levels in a higher energy electronic state (S_1; see Fig. B3.6.7). The energy in the vibrational levels is rapidly lost (~10^{-12} sec) to the surroundings as thermal energy, so that the excited chromophore is very largely in its ground vibrational level.

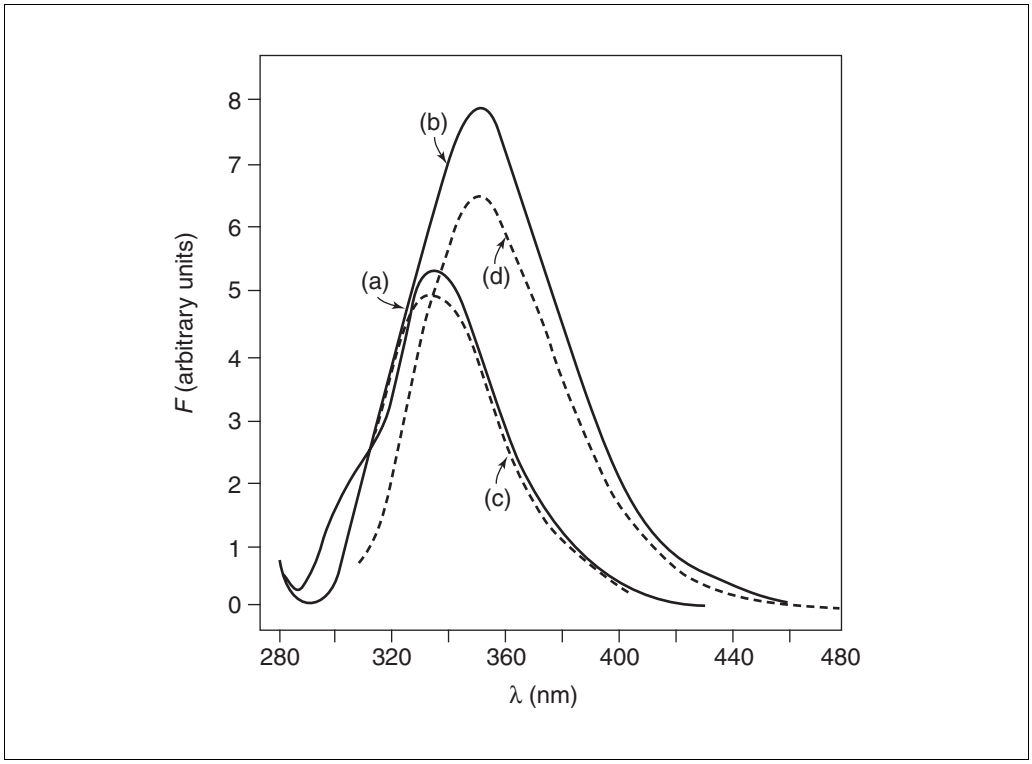

Figure B3.6.8 Quenching of the tyrosine fluorescence of β-lactoglobulin. Spectra were recorded in the absence (a, c) and presence (b, d) of 9.48 M urea, and excited at 275 nm (solid lines, tyrosine and tryptophan excited) and 297 nm (broken lines, tyrosine not excited). Parameters: protein concentration 16.5 μM in 0.025 M phosphate, 0.068 M NaCl, pH 6.2; λ_{ex} = 8 nm and λ_{em} = 9 nm;. Perkin-Elmer MPF 2A spectrofluorometer. Reprinted from Creamer (1995) with permission from the American Chemical Society.

Return to the ground electronic state can result in the emission of radiation of lower energy and hence longer wavelength (Figs. B3.6.1 and B3.6.3), or the energy can be dissipated in a number of ways that are nonradiative (Fig. B3.6.7), including quenching (see Quenching, below). The ratio of the rate constants for these two alternative processes defines the quantum yield or fluorescence efficiency, which is almost always less than unity.

The chromophores tyrosine and tryptophan absorb radiation in the 280 nm region, re-emitting a proportion of it as fluorescence. The quantum yields for tryptophan, tyrosine, and phenylalanine are 0.2, 0.1, and 0.04, respectively. Coupled with their relative absorption coefficients (*UNIT B1.3*), it can be seen why most protein fluorescence spectra are dominated by tryptophan.

Quantitation of the fluorescent properties of a protein can provide a number of rather specific parameters that allow assessment of the correct folding, besides giving additional structural information.

Tyrosine Fluorescence Is Frequently Not Seen in the Presence of Tryptophan

The fluorescence intensity of free tyrosine is about one-fifth of that of tryptophan, but in proteins it is usually much weaker still. This is due to a combination of interactions—such as hydrogen bonding to peptide carbonyl groups, the presence of neighboring charged groups, and nonradiative energy transfer to tryptophan or disulfide bonds—all of which can result in tyrosine fluorescence being diminished or quenched. The degree of quenching varies from protein to protein and can be observed by paying careful attention to bandwidths (see Basic Protocol 1). For instance, β-lactoglobulin contains two tryptophans and four tyrosines. In Figure B3.6.8, the fluorescence spectrum excited at 275 nm shows no sign of a band characteristic of tyrosine at 303 nm. In addition, λ_{max} is the same at 332 nm whether excitation is at 275 nm or 297 nm. (Note that, in the latter case, a correct spectrum cannot be obtained below ~310 nm owing to problems of bandwidth; see Basic Protocol 1, step 3). Thus, essentially all the tyrosine fluorescence is quenched (see Lakowicz, 1983) as seen for native β-lactoglobulin (Fig. B3.6.8). In the unfolded protein, the tyrosines and tryptophans are further apart and mobile, and little or no quenching takes place, as shown by the separate shoulder at 303 nm.

λ_{max} and I_{max} Reflect the Detailed Environment of Aromatic Residues

The precise wavelength of emission is, in the case of tryptophan, dependent on the polarity of the immediate environment of the chromophore in the protein, and maximum values have been observed over a broad range between 308 nm (highly nonpolar) and 352 nm (fully exposed to solvent). In β-lactoglobulin, the maximum emission intensity is at 332 nm, compared to 350 nm for the fully unfolded protein (Fig. B3.6.8). In tumor necrosis factor (TNF), λ_{max} is 320 nm (Fig. B3.6.9), showing that the tryptophan residues in this protein are, on average, packed in a less polar environment. The value of 336.5 nm for λ_{max} in acid solution indicates partial unfolding to a molten globule state. The value of λ_{max} therefore provides both a characteristic parameter for a particular protein and structural information concerning the average degree of burial of the tryptophans. The wavelength of tyrosine fluorescence, on the other hand, does not vary with polarity of environment and occurs with a maximum at 303 nm. Phenylalanine does not absorb above 275 nm, so that its weak fluorescence is not normally observed.

The intensity of fluorescence is, for both tyrosine and tryptophan, dependent upon interactions with neighboring groups that may quench the fluorescence emission from the native conformation. In solutions of denaturant, tryptophan and tyrosine fluorescence

are quenched, relative to the emission intensity in water. Depending on the degree of quenching of these chromophores in the folded protein, therefore, denaturation can result in an increase or a decrease in intensity, as well as a shift in λ_{max} to longer wavelengths (350 to 355 nm). In the case of TNF (Fig. B3.6.9), the large reductions in fluorescence intensity observed on unfolding the protein in guanidine or acid show that the tryptophan residues in the native protein are relatively unquenched. By contrast, tryptophan fluorescence in native β-lactoglobulin is seen to be considerably quenched relative to the denatured protein (Fig. B3.6.8). Although specific assignments of these interactions cannot be derived from the spectra, the intensity of fluorescence provides a further empirical characteristic of a protein. Note that caution should observed when comparing recorded values of λ_{max} and I_{max} (see Basic Protocol 1).

Quenching

Fluorescence quenching provides an indicator of the solvent accessibility of tryptophans and of the dynamics of protein conformation. The presence of certain extrinsic groups in physical contact with an excited chromophore can lead to sharing or transfer of the excitation energy, with consequent reduction in the quantum yield and diminution of fluorescence intensity. The efficiency of this quenching depends on the electronic structure of the extrinsic molecule or ion. Because it also depends on the access of the quencher to the chromaphore, a study of quenching can provide a further and independent probe of the packing of tryptophan in the protein. Iodide ion (I^-), cesium ion (Cs^+), and succinimide are useful quenchers for determining accessibility (a fuller list of quenchers for proteins is provided by Eftink and Ghiron, 1981). The ionic character of I^- and Cs^+ and the relatively large size of succinimide mean that these quenchers cannot readily penetrate

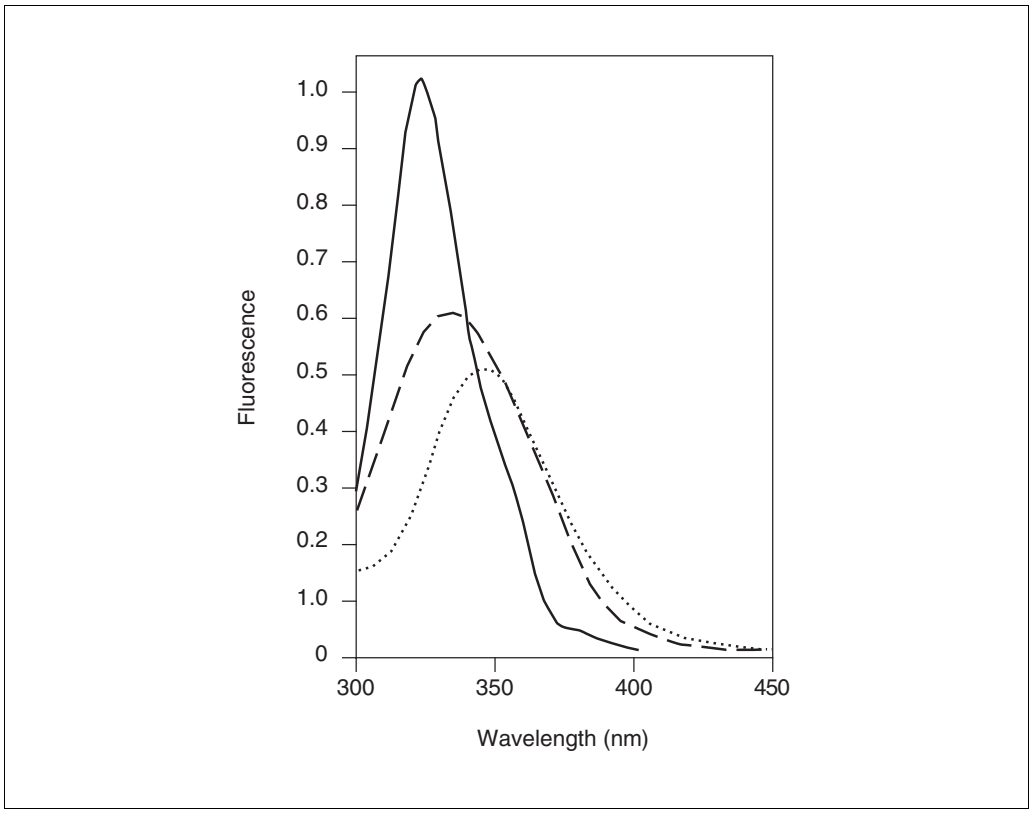

Figure B3.6.9 Quenching of fluorescence of tumor necrosis factor (TNF) on denaturation. Fluorescence emission spectra are shown for native TNF (solid line), guanidine-unfolded TNF (dotted line), and acid-denatured TNF (dashed line). Parameters: TNF concentration, 30 μg/ml; λ_{ex} = 280 nm; bandwidths ranging from 16 to 24 nm. Measurements made using Perkin-Elmer MPF3 spectrofluorimeter. Reprinted from Hlodan and Pain (1994) with permission of Elsevier Science.

the nonpolar interior of the protein, so they can be used to differentiate between buried and solvent-accessible chromophores. Their accessibility to an aromatic residue in the surface of the protein will depend, in addition, on the stereochemistry, chemical nature, and, in particular, charge of the surrounding groups. Provided that the assumptions are valid (i.e., the system is not too complex), quenching can be readily quantitated using the Stern-Volmer equations to give the quenching constant (K_{SV}) and the fraction of the initial fluorescence that is accessible to the quencher (see Basic Protocol 1).

The quenching of fluorescence of phosphoglycerate kinase (PGK) is shown plotted according to the Stern-Volmer equation (see Fig. B3.6.6A). The quenching levels off above 0.9 M succinimide and 0.6 M iodide, respectively, indicating that part of the fluorescence is unquenched by these quenchers. The different degrees of quenching by iodide and succinimide are due to the nature of the neighboring groups affecting access to the tryptophan. The plot of some of the data according to the modified Stern-Volmer equation (Fig. B3.6.6B) shows the fluorescence quenched by succinimide to be 42% of the total. The different degrees of quenching at 316 and 350 nm are due to the two tryptophans having substantially different values of λ_{max}, and hence different degrees of accessibility to solvent. Thus, taking into account the crystal structure of PGK, one of the

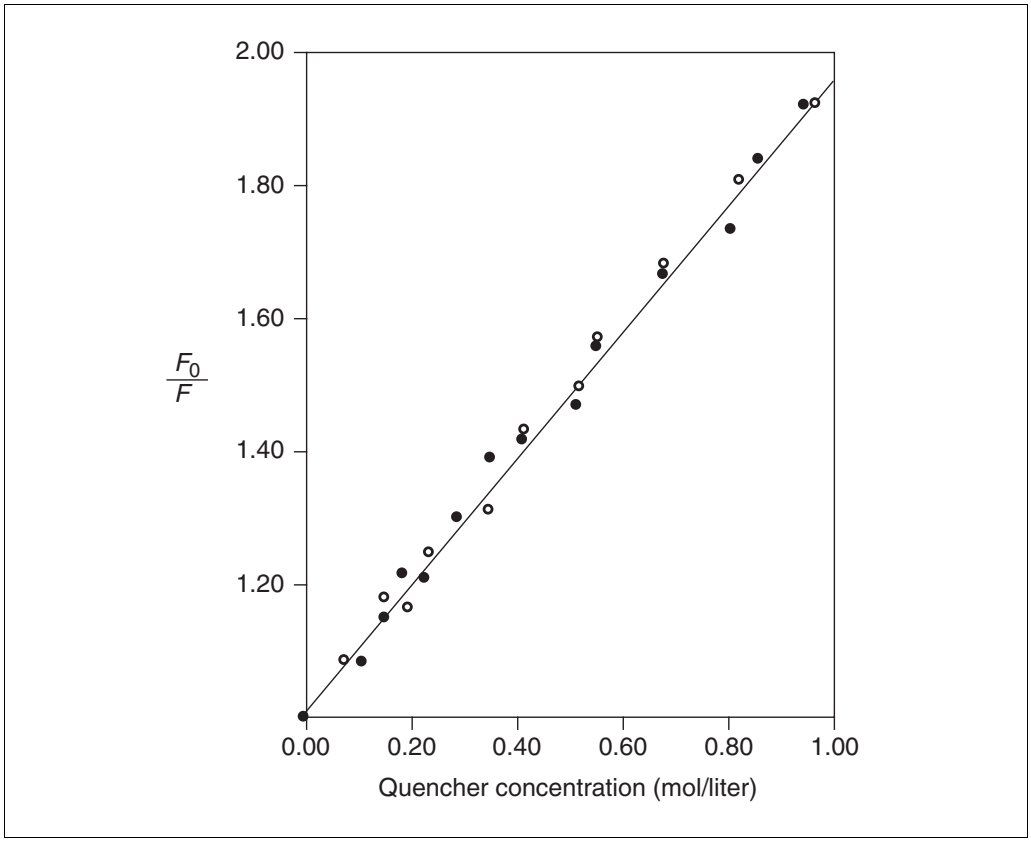

Figure B3.6.10 Assignment of fluorescence to trytophan W333 in 3-phosphoglycerate kinase (PGK). In the presence of 1.12 M succinimide (shown to quench the exposed tryptophan fluorescence; see Fig. B3.6.6), fluorescence is further quenched by acrylamide, which is able to gain access to the buried tryptophan. Fluorescence was measured at 316 nm (filled circles) and at 350 nm (open circles) and plotted according to the Stern-Volmer equation (see Basic Protocol 2, step 4). Fluorescence intensities were corrected for dilution and for quencher absorbance using ε_{295} = 0.236 for acrylamide. The linear plot indicates the quenching of a single residue. Measurements made using a Perkin-Elmer MPF3-L. Reprinted from Varley et al. (1991) with permission of Elsevier Science.

two tryptophans, W333, is seen to be buried and inaccessible to quencher while the other, W308, is quenched with a quenching constant of K_{SV} = 0.39 liters/mol.

Another quencher, acrylamide, can quench the fluorescence not only of exposed but also of buried tryptophan residues, based on its ability to diffuse through the protein structure, if the dynamics of the folded structure allow. The efficiency of quenching is, therefore, a reflection of the tightness of packing and the dynamics of the protein. Figure B3.6.10 shows the results of acrylamide quenching of the buried W333 of PGK (in this experiment the more exposed W308 is already quenched by the presence of succinimide). The linearity of the plot, combined with the coincidence of the quenching measured at 316 and 350 nm (in contrast to Fig. B3.6.6), shows that only one of the two tryptophans is being quenched by acrylamide. A quenching constant K_{SV} = 0.94 liters/mol is obtained from the slope. The use of quenchers in this manner can help in assigning the fluorescence of aromatic residues. In general, bimolecular rate constants for quenching (which reflect more directly the dynamics of the protein) can, with input from the three-dimensional structure, help to characterize the dynamics of the environment surrounding aromatic residues.

Ligand Binding

When a ligand binds specifically to its receptor protein, there is frequently a change in the fluorescence of either the ligand, if it is fluorescent, or the protein, if an aromatic group is close to the binding site. A good model ligand, anilinonaphthalene sulfonate (ANS), exhibits negligible fluorescence when free in solution but emits strongly when bound. In this way it acts as a good probe of nonpolar patches on the surface of a protein, as with bovine serum albumin. It has been used to detect partial unfolding in numerous proteins, and also to characterize the molten globule state (Christensen and Pain, 1994; Kuwajima and Arai, 2000).

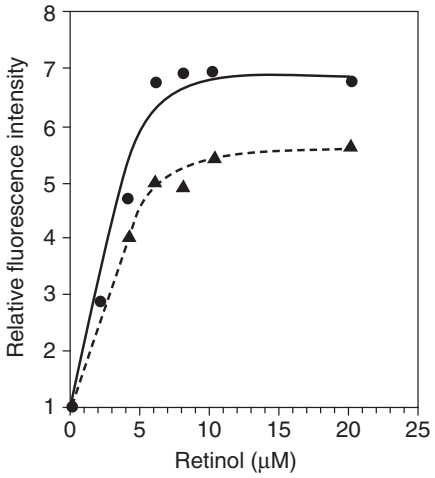

Figure B3.6.11 The binding of retinol to β-lactoglobulin that has been denatured by exposure to high pressure. The sample contaiining 270 μM β-lactoglobulin was pressurized to 400 MPa for 15 min. After release of pressure, retinol in ethanol was added and fluorescence was measured as a function of time. Parameters: final protein concentration 8.5 μM in 20 mM phosphate buffer; λ_{ex} = 330 nm; λ_{em} = 470 nm. Circles, native β-lactoglobulin; triangles, pressurized β-lactoglobulin. Reprinted from Ikeuchi et al. (2001) with permission from the American Chemical Society.

Many other ligands, however, have important physiological roles. Retinol is a vitamin A derivative that binds to β-lactoglobulin, which has been reported to enhance the intestinal uptake of retinol, triglyceride, and long-chain fatty acids in preruminant calves. Binding of retinol to β-lactoglobulin, monitored by fluorescence, has been used as a complementary probe to study the effect of pressure on the conformation, association, and gel formation of the protein (Ikeuchi et al., 2001). Typical binding curves are obtained, which show that β-lactoglobulin, which is reported to renature after exposure to high pressure, nevertheless loses a substantial proportion of its ability to bind retinol (Fig. B3.6.11).

These examples illustrate the fact that fluorescence can be used to follow the equilibria and kinetics of a wide variety of proteins and ligands, enzymes and substrates. The response of detectors for fluorescence is rapid and enables reactions to be followed at the subnanosecond level (Gruebele, 1999).

Fluorescence Depolarization

If a polarizer is placed in the excitation beam, the radiation passing through the sample solution will be plane-polarized. This beam will now be absorbed only by those chromophores whose transition dipoles are in the same plane (see Fig. B3.6.12). If the chromophore does not rotate within 10^{-8} sec (see Fig. B3.6.7), the fluorescence will be emitted

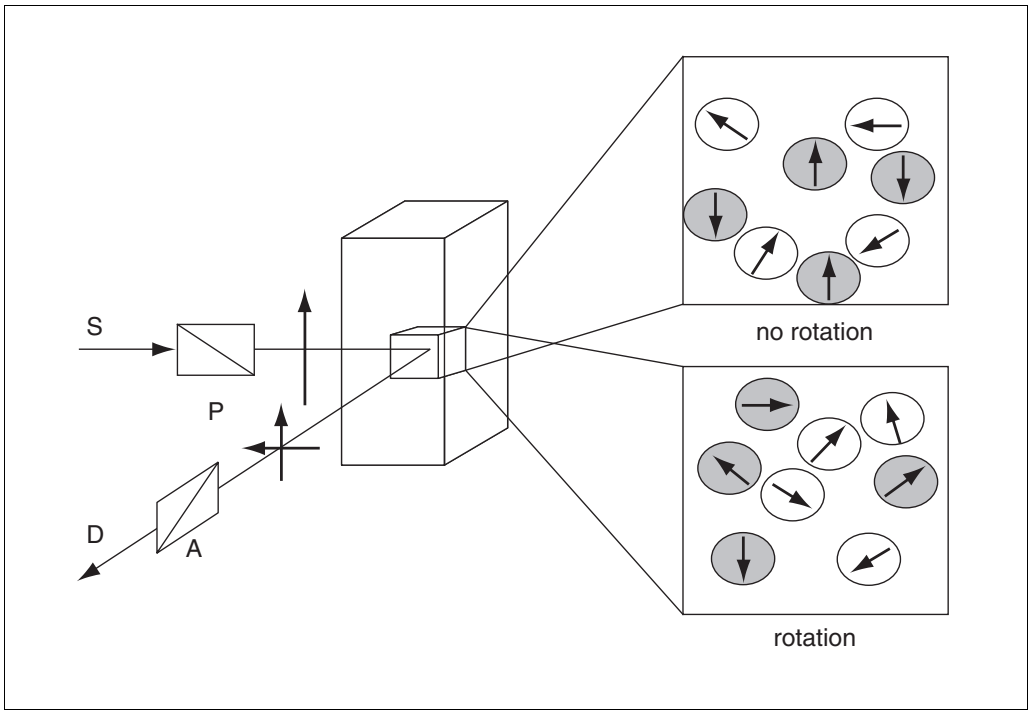

Figure B3.6.12 Depolarization of fluorescence indicates rotation of the chromophore. Monochromatic radiation from the source (S) has all but the vertically polarized electric vector removed by the polarizer (P). This is absorbed only by those molecules (see Fig. B3.6.5) in which the transition dipole of the chromophore is aligned vertically. In the case where these molecules do not rotate appreciably before they fluoresce (“no rotation”), the same molecules will fluoresce (indicated by shading) and their emitted radiation will be polarized parallel to the incident radiation. The intensity of radiation falling on the detector (D) will be zero when the analyzer (A) is oriented perpendicular to the polarizer. In the case where the molecules rotate significantly before fluorescence takes place, some of the excited chromophores will emit radiation with a horizontal polarization (“rotation”) and some with a vertical polarization. Finite intensities will be measured with both parallel and perpendicular orientations of the analyzer. The fluorescence from the remainder of the excited molecules will not be detected. The heavy arrows on the left of the diagram illustrate the case where there is rotation.

Table B3.6.1 Summary of the Interpretation of Fluorescence Spectra of Proteins[a,b]

1. All fluorescence of a protein is due to tryptophan, tyrosine, and phenylalanine, unless the protein contains another fluorescent component.
2. Maximum fluorescence of tryptophan occurs at lower wavelengths (<350 nm) and has greater intensity the lower the polarity of its environment.
   a. If λ_{max} is located at lower wavelengths when the protein is in a polar solvent—e.g., water—the tryptophan must be buried in a nonpolar environment.
   b. If λ_{max} is shifted to shorter wavelengths when the protein is transferred to a nonpolar medium, either the tryptophan is on the surface of the protein or the solvent induces a conformational change that causes it to be exposed to the solvent.
3. If a substance known to be a quencher quenches tyrosine and/or tryptophan fluorescence in a protein, these residues must be on the surface. If it fails to do so, there are several possible reasons:
   a. The residue may be buried in the interior of the protein.
   b. The residue may be in a crevice whose dimensions are too small for the quencher to enter.
   c. The residue may be in highly charged surroundings whose charge may repel a quencher with the same charge. In such a case, a quencher of the opposite charge or no charge would be expected to quench the fluorescence.
4. If a substance that does not affect the quantum yield of the free amino acid affects the fluorescence of a protein, it probably does so by inducing a conformational change in the protein.
5. If tryptophan or tyrosine are in a polar environment, their quantum yield decreases with increasing temperature, whereas in a nonpolar environment there is little change. Deviation from a monotonic decrease with increasing temperature signifies a change in polarity of the tryptophan environment, the sign of the change indicating the direction of change in polarity. Either indicates a conformational change in the protein.
6. Tryptophan fluorescence is quenched by protonated acidic groups, either in the neighboring environment or covalently attached as an α-carboxyl group. If the fluorescence changes with a particular pK, then a residue with that pK must be close to the tryptophan. Such an interpretation holds only if evidence is provided that no pH-induced conformational change is taking place.
7. If the binding of a substance is accompanied by quenching of tryptophan fluorescence, either there is a resulting conformational change or tryptophan is in or very near the binding site. Furthermore, because a decrease in polarity causes a shift to shorter wavelengths, a decrease in λ_{max} on binding indicates that water is excluded in the complex.
8. If the absorbance spectrum of a small molecule overlaps the emission spectrum of tryptophan and if the distance between them is small, quenching will be observed. If binding of such a molecule to a protein quenches tryptophan fluorescence, tryptophan must be in or near (<2 nm away from) the binding site.

[a]Adapted from Freifelder (1982). Reproduced with permission of W.H. Freeman, New York.

[b]In making these interpretations, two important facts must be borne in mind. First, fluorescence is so sensitive to environmental factors that alternative interpretations must always be considered. Second, if a protein contains more than one tryptophan, each may have a different quantum yield. For this reason the absolute magnitude of changes cannot be used to determine the fraction in a given environment.

with the same degree of polarization and will pass through a second polarizer (or analyzer) oriented parallel to the first polarizer. If, on the other hand, the excited chromophores rotate before fluorescence takes place, emission will take place from a smaller number of molecules reoriented with transition dipoles parallel and perpendicular to the exciting radiation (Fig. B3.6.12). The emitted radiation is now said to be depolarized. The polarization (P) can be quantitated as

$$P = (I_{par} - I_{perp})/(I_{par} + I_{perp})$$

where I_{par} and I_{perp}, respectively, are the intensities measured parallel and perpendicular to the original plane of polarization, usually vertical.

For chromophores that are part of small molecules, or that are located flexibly on large molecules, the depolarization is complete—i.e., $P = 0$. A protein of $M_r = 25$ kDa, however, has a rotational diffusion coefficient such that only limited rotation occurs before emission of fluorescence and only partial depolarization occurs, measured as $1 > P > 0$. The depolarization can therefore provide access to the rotational diffusion coefficient and hence the asymmetry and/or degree of expansion of the protein molecule, its state of association, and its major conformational changes. This holds provided that the chromophore is firmly bound within the protein and not able to rotate independently. Chromophores can be either intrinsic—e.g., tryptophan—or extrinsic covalently bound fluorophores—e.g., the dansyl (5-dimethylamino-1-naphthalenesulfonyl) group. More detailed information can be obtained from time-resolved measurements of depolarization, in which the kinetics of rotation, rather than the average degree of rotation, are measured. For further details, see Lakowicz (1983) and Campbell and Dwek (1984).

Although potentially powerful, the interpretation of fluorescence depolarization results requires extreme caution.

Table B3.6.1 provides a summary guide for interpreting results obtained using the techniques described in this unit.

In conclusion, given a protein in which tryptophan is partially or wholly buried, quantitative estimates of quenching by different quenchers provide a further fingerprint that is specific to the particular conformation of a recombinant protein and which—combined with other fluorescence, circular dichroism, and gel electrophoresis data—forms the basis for specific identification and quality assessment.

COMMENTARY

Background Information

Fluorescence depends on the atomic environment of the tryptophan and tyrosine residues and on their accessibility to solvent. Although the structural information is limited strictly to the immediate neighborhood of the relatively few aromatic residues, the cooperative nature of protein conformations means that changes in aromatic fluorescence frequently reflect more widespread changes in conformation. Similarly, measures of solvent accessibility obtained from fluorescence quenching reflect changes in the folding and packing of nonaromatic residues in a very sensitive way. Along with its sensitivity to small changes in conformation, fluorescence offers the additional advantage of requiring very small amounts of protein, in favorable cases down to tens of micrograms.

The method is less powerful in characterizing proteins lacking tryptophan, for reasons mentioned earlier (see Support Protocol), and does not apply at all to the rare proteins that lack tyrosine as well. Near-UV circular dichroism measurements (*UNIT 7.6*) can still be of considerable value for proteins containing tyrosine and, in both cases, both far-UV circular dichroism and urea-gradient gel electrophoresis (*UNIT 7.4*) provide powerful contributions to characterization.

Extrinsic fluorescent probes can provide additional useful information about the integ-

rity of protein conformation. 8-{2-[(Iodoacetyl)ethyl]amino} naphthalene sulfonic acid (ANS) fluoresces only weakly in free solution but strongly when bound to a nonpolar surface. Originally used as a competitive probe to monitor fatty-acid binding to albumin, it has been widely used in studies of protein conformation—in partially denatured states at equilibrium as well as in transient intermediates that accumulate during protein folding (reviewed by Christensen and Pain, 1994). Fully folded native proteins in general bind only limited amounts of ANS, although these are sufficient to characterize the surface hydrophobicity of a number of proteins (Cardamone and Puri, 1992). Fully unfolded proteins do not bind ANS. Partially folded or partially unfolded proteins will, however, bind ANS—indicating the presence of associated nonpolar groups that constitute a surface site, or the existence of relatively loosely packed hydrophobic cores into which ANS can intercalate. This and similar probes are mainly used as empirical indicators of conformational change, as the detailed mechanisms of interaction are not well defined.

A good example of its use applied to a protein associated with food chemistry is that of β-lactoglobulin. Ikeuchi et al. (2001) used ANS as a probe to follow β-lactoglobulin denaturation under high pressure and its subsequent renaturation on release of pressure (Fig. B3.6.11). The denaturation was shown to be completely reversible at pH 2 but not at neutral pH, explaining why whey protein isolates subjected to high pressures form a gel at pH 7 but not at acid pH.

The binding of fluorescent probes of different hydrophobicities can be used to compare the surface properties of proteins in relation to their physicochemical properties, such as foaming and emulsifying. CPA (*cis*-parinaric acid) has been used to study eleven food-related proteins, and the results have been compared with theoretical models for predicting foam capacity (Arteaga and Nakai, 1993; also see *UNIT B5.2*).

Fluorescence forms the basis of a wide variety of assays, particularly those that are immunologically based. IgG has been quantitated in bovine milk using a solid-phase agglutination assay using fluorescein isothiocyanate (FITC)–labeled rabbit anti–bovine IgG as the detection antibody (Losso et al., 1993). Such assays are both repeatable and rapid.

Fluorescence energy transfer, which can be observed when the absorbance spectrum of a fluorescent probe such as the dansyl group overlaps the emission spectrum of tryptophan, provides a means of estimating distances between groups, and hence of conformation and conformational change. An example where this approach is used alongside ANS fluorescence to follow protein folding is reported by Agashe et al. (1995).

In the present context, fluorescence is an important tool for the rapid comparison of a protein with a standard sample of the recombinant or the authentic protein, as part of the process of characterizing and authenticating a recombinant protein. Coupled with measurements of the depolarization of fluorescence, the method can provide information on the dynamics of a protein and on the change in dynamics in response to, for example, binding of ligands.

Critical Parameters

Recording a spectrum is basically very simple, but the critical element is to avoid the production of artifacts. For this reason, it has to be emphasized that scrupulous attention to the cleanliness of cells and of the cell compartment of the spectrofluorometer is essential, as is attention to the clarification of solutions and buffers (see Strategic Planning).

It is important also to make intelligent choices in the selection of instrumental parameters to optimize resolution and signal-to-noise ratio (see Basic Protocol 1).

Troubleshooting

One of the main causes of artifacts in the spectroscopy of proteins is the frequent tendency of proteins to aggregate, either during the folding step in recovery from inclusion bodies or as a result of the delicate balance of solubility of many proteins. For this reason, it is important to pay strict attention to monitoring aggregation (see Strategic Planning, discussion of Clarification of Solutions).

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Key Reference

Lakowicz, J.R. 1983. See above.

An essential text, providing a thorough, but clear and readable description of the practice and interpretation of fluorescence spectroscopy, with particular reference to proteins.

Contributed by Roger H. Pain
Jozef Stefan Institute
Ljubljana, Slovenia

Overview of Protein Purification and Characterization

AIMS AND OBJECTIVES

Protein purification has a >200-year history: the first attempts at isolating substances from plants having similar properties to "egg albumen," or egg white, were reported in 1789 by Fourcroy. Many proteins from plants were purified in the nineteenth century, though most would not be considered pure by modern standards. A century later, ovalbumin was the first crystalline protein obtained (by Hofmeister in 1889). The year 1989 may not go down in history as a milestone in protein chemistry, but since then there has been a resurgence of interest in proteins after more than a decade of gene excitement.

The aims of protein purification, up until the 1940s, were simply academic. To then, even the basic facts of protein structure were not fully appreciated, and pure proteins were needed just to study structure and test the rival theories of the pre-DNA days. During the Second World War, an acute need for blood proteins led to development of the Cohn fractionation procedure for purification of albumin and other proteins from serum (Cohn et al., 1946). This was the inception of large-scale protein purifications for commercial purposes; Cohn fractionation continues to be used to this day.

As more proteins, and particularly enzymes, were purified and crystallized, they started to be used increasingly in diagnostic assays and enzymatic analyses, as well as in the large-scale food, tanning, and detergent industries. Many enzymes used in industry are not in fact very pure, but as long as they do the job, that is sufficient. "Process" enzymes such as α-amylase, proteases, and lipases are produced in ton quantities, mainly as secretion products in bacterial cultures, and may undergo only limited purification processes to minimize costs. Enzymes have long been used commercially in the food industry. Products such as cheese and high-fructose corn syrup could not be produced without the addition of enzymes. Rennet, used in cheesemaking, was one of the first commercially available bioengineered proteins. Partially purified protein preparations, such as whey or soy protein concentrates or isolates, are added as functional ingredients to a variety of food systems. At the other extreme, enzyme products for research and analysis require a high degree of purification to ensure that contaminating activities do not interfere with the intended use. Anyone familiar with molecular biology enzymes will appreciate how minute levels of contamination of DNase or RNase can completely destroy carefully planned experiments.

It is often necessary to purify a particular protein to better understand its role in the nutritional value and physicochemical properties of food. Similarly, many enzymes have been purified to study their effect on the texture, color, flavor, and nutritional value of foods. The purification and characterization of protein-based microbial toxins has been necessary to better understand their mechanisms of action and their roles in food-borne disease.

The 1960s and 1970s could be described as the peak years for protein and enzyme research, and most of the methods used in protein purification were established by then, at least in their principles. More recent developments have been mainly in instrumentation designed to optimize the application of each methodology. Developments in instrumentation have been stimulated by the rapid progress in molecular biology, because gene isolation has often been preceded by isolation of the gene product. Because such products can now be characterized sufficiently (i.e., partially sequenced) using minute amounts of protein, the need for large-scale or even moderate-scale procedures has decreased. Hence there has been an explosive development of modern equipment designed specifically for dealing with milligram to microgram amounts of protein. On the other hand, structural studies using X-ray crystallography and nuclear magnetic resonance (NMR) may require hundreds of milligrams of pure protein, so larger-scale equipment and procedures are still needed in the research laboratory. In the food industry, there are many applications for large-scale purifications (e.g., amylase, protease, and lipase enzyme preparations; fractionated milk protein preparations such as β-lactoglobulin and α-lactalbumin) and small-scale purifications (e.g., myosin and myoglobulin, lipoxygenase, lysozyme, and antibodies, just to name a few).

Many proteins occur in minute amounts in the natural source, and their purification can be a major task. Heroic efforts in the past have used kilogram quantities of starting materials, and

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ended up with a few micrograms of pure product. It is now more usual, however, to take the genetic approach: clone the gene before the protein has been isolated or even properly identified, then express it in a suitable host cell culture or organism. The expression level may be orders of magnitude higher than in the original source, which will make purification a relatively simple task. It can be useful to know beforehand some physical properties of the protein, to facilitate the development of a suitable purification protocol from the recombinant source. On the other hand, there are now several ways of preparing fusion proteins, which can be purified by affinity techniques without any knowledge of the properties of the target protein. Moreover, there are ways of modifying the expressed product to simplify purification further.

Thus, the approach to protein purification must first take into account the reason it is being done, as the methods will vary greatly with different requirements. At one extreme is the one-of-a-kind purification, in a well-financed and equipped laboratory, that is carried out to obtain a small amount of product for sequencing so that gene isolation can proceed. In this case, expense of equipment and reagents may be no problem, and a very low overall recovery of product can be acceptable, provided it is pure enough. At the other extreme are the requirements of commercial production of a protein in large amounts on a continuing basis, where high recovery and economy of processing are the chief parameters to be considered. There are many intermediate situations as well.

Many publications in the area of protein research are entitled "Purification and characterization of…," and describe a purification procedure in sufficient detail that it can be reproduced in another laboratory. The characterization section may include structural, functional, and genetic information, and carrying out such studies is likely to require at least milligram quantities of pure protein. Ideally the purification should involve a small number of steps, with good recovery at each step. If the recovery is poor (<50% at any step), however, there should be some indication of what happened to the missing activity. Has it been discarded in the other fractions for the sake of purity, or does it represent a true loss of activity? If the latter, then the end-product may be less than fully active despite apparent homogeneity indicated by standard analysis. The choice between recovery and purification at each step can be problematic; taking a narrow cut of a chromatographic peak may provide a very pure fraction at the expense of losing a good deal of less pure active component on either side. In making such decisions, the objective of the exercise must be kept in mind: if yield is not important, then the choice of poor yield for the sake of purity may be logical.

By far the most important requirement of a publication is reproducibility of the method reported. It is not sufficient to have carried out the process only once if it is expected that other investigators will want to repeat it. There are always factors that influence the process that may be overlooked at first, and which can have a major effect on the purification procedure if varied slightly. The reported process should always be repeated exactly as described before submitting the manuscript for publication.

This unit discusses protein purification and characterization in very general terms. Many of the procedures mentioned here are discussed in greater detail in the companion volume, *Current Protocols in Protein Science* (Coligan et al., 2001).

SOURCES OF MATERIAL FOR PROTEIN PURIFICATION

For many people embarking on a protein purification project, there is no choice of material. They are studying a particular food type, biological tissue, or organism, and the objective is to purify a protein from that source. However, there may be approaches that can make the project simpler. If, for instance, the source is difficult to obtain in large amounts, it may be best to carry out preliminary trials on a source species more readily obtained. Once a protocol for purifying the protein from substitute sources has been worked out, it will be much easier to develop one using the desired material—the identical procedure may work satisfactorily. Proteins differ to a fairly small extent between species that have diverged within about 100 million years, a time frame that groups together most higher mammals. Thus the behavior of proteins derived from different animals with respect to the various fractionation procedures is likely to be similar, and a protocol worked out for bovine or porcine tissues is likely to need only minor adjustments for application to another species.

A second example is where the interest is mainly on the function of a protein, especially an enzyme, for which functions and actions have generally been strongly conserved through evolution. In that case, a preliminary screening of potential sources or, better still, the

literature should provide a raw material that is best suited to the investigator's purposes. Considerations should include the following: (1) What functions are required of the end product? For instance, an enzyme having a low K_m may be needed, so selecting the source with the highest activity may not suffice. (2) How convenient is it to grow or obtain the raw material, and are there problems concerning pathogenicity or extractability? (3) Does the quantity of the protein vary with growth conditions or age, and does it deteriorate in situ if left too long? Obviously one requires a source that reliably produces the highest amount of the desired protein per unit volume to maximize the chances of developing a good purification procedure. (4) What storage conditions are required for the raw material? It is important to consider that fresh raw material may not be immediately available whenever a purification is attempted.

The above considerations are relevant to the traditional situation for commencing a protein purification project. It is becoming increasingly common, however, for proteins to be purified as recombinant products using techniques in which the gene is expressed in a host organism or in cultured cells. This of course requires the gene encoding the protein of interest to be available. Until the mid-1980s, such material was usually obtained by hybridization of an oligonucleotide synthesized according to amino acid sequence information. This required the protein to have been purified first, so the initial task of protein purification still needed to be done at least once. More recently, genetic techniques have permitted the isolation of many genes encoding known proteins, even though the proteins may never have been studied directly. Moreover, with the expansion of the Human Genome Project and related DNA sequencing efforts, many genes for both known and unknown proteins will become available and will be able to be expressed in recombinant form without ever being purified from the host species. As a result some completely new considerations for protein purification come into play, including the possibility of modifying the gene structure not only to increase expression level and alter the protein product itself to enhance a desired function, but equally importantly to aid in purification. Recombinant proteins may be expressed in bacteria, yeasts, and animal tissue cultures.

DETECTION AND ASSAY OF PROTEINS

During a protein purification procedure there are two measurements that need to be made, preferably for each fraction. Measurements of the amount of total protein and of the amount (usually bioactivity) of the desired protein both must be made. It is not possible to isolate a protein without a method of determining whether it is present; an assay, either quantitative or at least semiquantitative, indicating which fraction contains the most of the desired protein is essential.

Assays may range from the quick-and-easy type (e.g., instantaneous spectrophotometric measurement of enzyme activity) to long and tedious bioassays that may take days to produce an answer. The latter situation is very difficult, because by the time one knows where the protein is, it may be "was," owing to degradation or inactivation. Moreover, this may not become clear until the next step has been completed and its products assayed. Any assay that is quick is therefore advantageous, even if it means a sacrifice of accuracy for speed.

Measurement of total protein is useful, as it indicates the degree of purification at each step. However, unless the next step critically depends on how much protein is present, total protein measurement is not extremely important: a small sample can be put aside and measured later, when the purification is complete. It is, however, very important to know how much protein is present in the final, presumed pure sample, as this will indicate the specific activity (if the protein has an activity), which can be compared with other preparations. The general object is to obtain as high a specific activity as possible (taking into account recovery considerations), which means retaining as much of the desired protein as possible while ending up with as little total protein as possible.

METHODS FOR SEPARATION AND PURIFICATION OF PROTEINS

The methods available for protein purification range from simple precipitation procedures used since the nineteenth century to sophisticated chromatographic and affinity techniques that are constantly undergoing development and improvement. Methods can be classified in several alternative ways—perhaps one of the best is based on the properties of the proteins that are being exploited. Thus the methods can be divided into four distinct but interrelated groups depending on protein

characteristics: surface features, size and shape, net charge, and bioproperties.

Methods Based on Surface Features of Proteins

Surface features include charge distribution and accessibility, surface distribution of hydrophobic amino acid side chains, and, to a lesser extent, net charge at a given pH (see discussion of net charge). Methods exploiting surface features mainly depend on solubility properties. Differences in solubility result in precipitation by various manipulations of the solvent in which the proteins are solubilized. The solvent, nearly always water containing a low concentration of buffer salts, can be treated to alter properties such as ionic strength, dielectric constant, pH, temperature, and detergent content, any of which may selectively precipitate some of the proteins present. Conversely, proteins may be selectively solubilized from an insoluble state by manipulation of the solvent composition. The surface distribution of hydrophobic residues is an important determinant of solubility properties; it is also exploited in hydrophobic chromatography, both in the reversed-phase mode and in aqueous-phase hydrophobic-interaction chromatography.

Also included in this group is the highly specific technique of immunoaffinity chromatography, in which an antibody directed against an epitope on the protein surface is used to pull out the desired protein from a mixture.

Methods Based on Whole Structure: Protein Size and Shape

Although the size and shape of proteins can have some influence on solubility properties, the chief method of exploiting these properties is gel-filtration chromatography. In addition, preparative gel electrophoresis makes use of differences in molecular size. Proteins range in size from the smallest classified as proteins rather than polypeptides, around 5000 Da, up to macromolecular complexes of many million daltons. Many proteins in the bioactive state are oligomers of more than one polypeptide (see below), and these can be dissociated, though normally with loss of overall structure. Thus many proteins have two "sizes": that of the native state, and that (or those) of the polypeptides in the denatured and dissociated state. Gel-filtration procedures normally deal only with native proteins, whereas electrophoretic procedures commonly involve separation of dissociated and denatured polypeptides.

Methods Based on Net Charge

The two techniques that exploit the overall charge of proteins are ion-exchange chromatography (by far the most important) and electrophoresis (e.g., *UNIT B3.1*). Ion exchangers bind charged molecules, and there are essentially only two types of ion exchangers, anion and cation. The net charge of a protein depends on the pH—positive at very low pH, negative at high pH, and zero at some specific point in between, termed the isoelectric point (pI). It should be stressed that at the pI a protein has a great many charges; it just happens that at this pH the total negatives exactly equal the total positives. The most charged state (disregarding the charge sign) is in the pH range 6.0 to 9.0. This is the most stable pH range for most proteins, as it encompasses common physiological pH values. Ion exchangers consist of immobilized charged groups and attract oppositely charged proteins. They provide the mode of separation that has the highest resolution for native proteins. High-performance reversed-phase chromatography has equivalent or even better resolution, but it generally involves at least partial denaturation during adsorption and so is not recommended for sensitive proteins such as enzymes. Protein purification using ion-exchange chromatography has mainly employed positively charged anion exchangers, for the simple reason that the majority of proteins at neutral pH are negatively charged (i.e., have a low isoelectric point).

Methods Based on Bioproperties (Affinity)

A powerful method for separating the desired protein from others is to use a biospecific method in which the particular biological property of the protein is exploited. The affinity approach is limited to proteins that have a specific binding property, except that proteins are theoretically able to be purified by immunoaffinity chromatography, which is the most specific of all affinity techniques. Most proteins of interest do have a specific ligand: enzymes have substrates and cofactors, and hormone-binding proteins and receptor molecules are designed to bind specifically and tightly to particular hormones and other factors. Immobilization of the ligand to which the protein binds (or of antibody to the protein) enables selective adsorption of the desired protein in the technique known as affinity chromatography. There are also nonchromatographic modes of exploiting biospecific interactions.

CHARACTERIZATION OF THE PROTEIN PRODUCT

Once a pure protein is obtained, it may be employed for a specific purpose, such as enzymatic analysis (e.g., glucose oxidase and lactate dehydrogenase), or as a therapeutic agent (e.g., insulin and growth hormone). However, it is normal, when a protein has been isolated for the first time, to characterize it in terms of structure and function. Several features are generally expected in characterization of a new protein. These include molecular weight, or at least the size of the subunit(s), determined by SDS-polyacrylamide gel electrophoresis and/or gel filtration. Spectral properties such as the UV spectrum (based on Trp and Tyr content), circular dichroism (CD) spectrum (secondary structure), and special characteristics of proteins with prosthetic groups (e.g., quantitation and spectra) may be presented. The quantity and nature of carbohydrates on glycoproteins should be determined. Also, if the gene has not already been reported, some amino-terminal sequence analysis should be given, if at all possible, along with the results of a database search for similar sequences. Functional proteins should be demonstrated to have the appropriate function, and for enzymes detailed kinetic characterization is appropriate. Ultimately the full three-dimensional structure of the protein may be determined, which will require crystals: any successful crystallization attempts should be reported.

THE PROTEIN PURIFICATION LABORATORY

The requirements for a protein purification laboratory cannot be exactly formulated because they depend greatly on the types and amounts of proteins being isolated. To cover all eventualities, it would be necessary to have one set of equipment to deal with submicrogram quantities and another set to deal with multigram quantities—a range of around 10^8! One laboratory dedicated to protein purification may not need small-scale equipment if, for example, it works with plasma proteins that are always available in large quantities. Another may have all the latest in high-performance equipment but not be able (nor need) to handle quantities of protein in excess of a few milligrams.

If it is assumed that neither extreme in quantity is to be attempted, and that the laboratory is handling a variety of protein types and sources, then certain basic pieces of equipment are needed. Obtaining the starting material and making an extract of it require homogenization equipment and centrifuges to remove insoluble residues. Preliminary fractionation, when starting with a crude extract of tissue or cells, requires equipment and materials that will not become clogged by particulates. Adsorbents and similar materials used at the first step should be relatively inexpensive so that when performance falls off after a few uses, owing to intransigent impurity buildup, they can be discarded. It is also relevant that a larger amount is handled at the initial step than later steps; therefore, reagent expense can be an important consideration. After the first one or two steps, the sample should be sufficiently clean and clear to enable use of high-performance equipment.

High-performance liquid chromatography, or HPLC, is a term with a variety of meanings. To some it refers exclusively to reversed-phase chromatography; to others it includes all sorts of chromatography provided that the equipment is fully automated and high-performance adsorbents are used. A high-performance system designed specifically for proteins—introduced by Pharmacia Biotech and called Fast Protein Liquid Chromatography, or FPLC—uses standard protein chromatographies such as ion exchange, hydrophobic interaction, and gel filtration. Scaleup is possible with larger equipment based on the FPLC design, so that laboratory development can be quickly translated to large-scale production. FPLC is designed to separate proteins in their native active configuration, whereas reversed-phase HPLC often causes at least transient denaturation during adsorption and elution. Reversed-phase HPLC has a high resolving power, but it is best suited to peptides and proteins smaller than ~30 kDa. Chromatography run with older-style low-pressure adsorbents is sometimes referred to as "low-performance" or "open-column" chromatography; neither of those descriptions is necessarily accurate. Simple fraction collector and monitoring equipment is needed. This equipment will be used for larger-scale operations (tens of milligrams of protein and upward), probably at an earlier stage in the protocol than with HPLC.

Various columns, both prepacked with proprietary adsorbents and empty for self-packing, will be needed, with the sizes and types depending on the scale of operation. Several anion-exchange columns (different sizes), one or two cation-exchange columns, and gel-filtration media are essential, along with a range of alternative adsorbents such as hydrophobic interac-

Table B4.1.1 Classification of Proteins by Structural Characteristics

Structural characteristic	Examples	Comments
Monomeric	Lysozyme, growth hormone	Usually extracellular; often have disulfide bonds
Oligomeric		
Identical subunits	Glyceraldehyde-3-phosphate dehydrogenase, catalase, alcohol dehydrogenase, hexokinase	Mostly intracellular enzymes; rarely have disulfide bonds
Mixed subunits	Aspartate carbamoyltransferase, pertussis toxin	Allosteric enzymes; different subunits have separate functions
Membrane-bound		
Peripheral	Mitochondrial ATPase, alkaline phosphatase	Readily solubilized by detergents
Integral	Porins, cytochromes P_{450}, insulin receptor	Require lipid for stability
Conjugated	Glycoproteins, lipoproteins, nucleoproteins	Many extracellular proteins contain carbohydrate

tion materials, dyes, hydroxyapatite, and chromatofocusing and specialist affinity media.

Fully equipped protein purification laboratories should also have preparative electrophoresis and isoelectric focusing apparatuses for rare occasions when other techniques fail to give sufficient separation.

In addition to equipment used in the actual fractionation processes, a variety of other items are needed. In particular it should be possible to change buffers quickly and to concentrate protein solutions with ease. These operations require such things as dialysis membranes, ultrafiltration cells, and gel-exclusion columns of various sizes.

Finally, equipment for assaying and analyzing the preparations is needed. Most such equipment is fairly standard in biochemical laboratories and includes spectrophotometers, scintillation counters, analytical gel and capillary electrophoresis apparatuses, immunoblotting materials, and immunochemical reagents.

CLASSIFICATION OF PROTEINS

Proteins can be classified in several different ways, such as by function, by structure, or by physicochemical characteristics. Each protein species consists of identical molecules with exactly the same size, amino acid sequence, and three-dimensional shape. In this way a solution of a mixture of proteins differs from a solution of synthetic polymers or sheared DNA, both of which contain a complete spectrum of possible sizes centered around the average. The protein mixture has only discrete sizes of molecules corresponding to each type of protein present. Although we could classify proteins by size, it would be of limited use, as there is usually no obvious relationship between size and function.

A more useful structural classification takes into consideration shape and oligomeric structure (Table B4.1.1). In part, structure reflects biological location and origin. Simple, fairly rigid protein molecules occur in the extracellular environment, more complex and readily deactivated molecules are found intracellularly, and hydrophobic proteins are associated with membranes.

Classification by function is more relevant (Table B4.1.2). Proteins can simply be stores of amino acids, can be structural, or can have specific binding functions. The most "functional" proteins are enzymes, which have both binding and catalytic roles. In part, this reflects the degree to which the detailed structure is a requirement for the protein's function, which in turn relates to conservation of structure through evolution. But as with every attempt at classification, there are going to be examples that do not fit the pattern well. Most proteins of interest to the pharmaceutical industry belong to the general class of binding proteins, for instance, hormones [e.g., insulin and bovine somatostatin (BST)], viral antigens (e.g., hepatitis B antigen), growth factors [e.g., interfer-

Table B4.1.2 Classification of Proteins by Function

Function	Examples
Amino acid storage	Seed proteins (e.g., gluten), milk proteins (e.g., casein)
Structural	
Inert	Collagen, keratin
With activity	Actin, myosin, tubulin
Binding	
Soluble	Albumin, hemoglobin, hormones
Insoluble	Surface receptors (e.g., insulin receptor), antigens (e.g., viral coat proteins)
With activity	Enzymes, membrane transporters (e.g., amino acid uptake systems, ion pumps)

ons, interleukins and colony-stimulating factors (CSFs)], and antibodies.

STRATEGIES FOR PROTEIN PURIFICATION

Soluble Extracellular Proteins

The source of soluble extracellular proteins is the extracellular medium, whether it be an animal source such as blood or spinal fluid, or a culture medium in which bacterial, fungal, animal, or plant cultures have been grown. Generally, these do not contain a large number of different proteins (blood is an exception), and the desired protein may be a major component, especially if produced as the result of recombinant expression. Nonetheless, the protein in the starting material may be quite dilute, and a large volume may therefore need to be processed. The starting fluid may also contain many compounds other than proteins, whose behavior must be taken into account. The first stage should aim mainly to reduce the volume and get rid of as much nonprotein material as possible; some protein-protein separation is also useful, but not essential. No general rules can be given, but a batch adsorption method using an inexpensive material such as hydroxyapatite, ion-exchange resin, immobilized metal affinity chromatography (IMAC) medium, or affinity adsorbent is best, if feasible. Following the first step, the sample should be in a form that is amenable to standard purification processes such as precipitation and column chromatography.

Intracellular (Cytoplasmic) Proteins

To obtain soluble intracellular proteins (which are mainly enzymes), cells must be broken open or lysed to release their soluble contents. The ease with which cell disruption can be accomplished varies considerably; animal cells are readily broken, as are many bacteria, but plants and fungi have tough cell walls. The macromolecular soluble contents of cells are mainly proteins, with nucleic acids as a minor but significant component. Bacterial extracts may be viscous unless DNase is added to break down the long DNA molecules. Although chromatographic procedures can be applied to crude extracts, valuable high-performance materials should not be employed in the first step, as there are always compounds, including unstable proteins, that may bind to them and be difficult to remove.

Membrane-Associated Proteins

There are two approaches to isolating a membrane-associated protein. In one method, the relevant membrane fraction can first be prepared and then used to isolate the protein. Alternatively, whole tissue can be subjected to an extraction that solubilizes the membranes and releases the cytoplasmic contents as well. The former is much better in that purification is accomplished by isolating the membranes: the specific activity of the solubilized membrane fraction will be much higher than in the second method. However, the process of purifying the membrane fraction may lead to substantial losses, and it may be difficult to scale up. If total recovery of the protein is more important than purity, a whole-tissue extract is likely to be more appropriate. Although this

means that a greater degree of purification is needed, the fact that membrane proteins have, by definition, properties somewhat different from those of cytoplasmic proteins permits some very effective purification steps (e.g., hydrophobic chromatography or fractional solubility separation).

Peripheral membrane proteins are only loosely attached and may be released by gentle conditions such as high pH, EDTA, or low (nonionic) detergent concentrations. Once in solution, some peripheral proteins no longer require the presence of detergent to maintain their solubility. Integral membrane proteins are much more difficult—they require high concentrations of detergent for solubilization (i.e., complete solubilization of the membrane is needed to release them) and generally are neither soluble nor stable in the absence of detergent. It is sometimes necessary to maintain natural phospholipids in association with the proteins in order to maintain activity. Even though the final objective may not require activity (e.g., amino acid sequencing), there is a need for some sort of assay during the purification process to determine where the protein is. If a particular band on a gel is known to be the desired protein, then no other assay is needed and loss of bioactivity can be allowed.

Purification processes may be affected by the presence of detergents. The problem of association with detergent micelles makes purifying integral membrane proteins difficult; the close association of the different proteins originating from membranes often results in very poor separation in conventional fractionation procedures.

Insoluble Proteins

Natural proteins that are insoluble in normal solvents are generally structural proteins, which are sometimes cross-linked by posttranslational modification. The first stage of purification is obvious—it involves extracting and washing away all proteins that are soluble, leaving the residue containing the desired material. Further purification in a native state, however, may be impossible; extracting away other proteins using more vigorous solvents or attempting to solubilize the target protein may destroy the natural structure. Cross-linked proteins such as elastin or aged collagen cannot be dissolved without breaking the cross-links, and different proteins may even be cross-linked together.

Insoluble Recombinant Proteins (Inclusion Bodies)

A major class of insoluble proteins are recombinant proteins expressed (usually in *Escherichia coli*) as inclusion bodies. These are dense aggregates found inside cells that consist mainly of a desired recombinant product, but in a nonnative state. Inclusion bodies may form for a variety of reasons, such as insolubility of the product at the concentrations being produced, inability to fold correctly in the bacterial environment, or inability to form correct, or any, disulfide bonds in the reducing intracellular environment. Their purification is simple, since the inclusion bodies can be separated by differential centrifugation from other cellular constituents, giving almost pure product; the problem is that the protein is not in a native state, and is insoluble. Some methods for obtaining an active product from inclusion bodies are described in Coligan et al. (2001).

Soluble Recombinant Proteins

Recombinant proteins that are not expressed in inclusion bodies either will be soluble inside the cell or, if using an excretion vector, will be extracellular (or, if *E. coli* is the host, possibly periplasmic). They can be purified by conventional means. In some systems, expression is so good that the desired product is the major protein present and its purification is relatively simple. In systems where the expression level is low, the purification process can be tedious, though easier, it is hoped, than isolation from the natural source. It should be remembered that a procedure developed for isolating a protein from natural sources may not work successfully with the recombinant product, because the nature of the other proteins present influences many fractionation procedures.

Because of the difficulties often experienced in purifying recombinant proteins, a variety of vector systems (for examples, see Sassenfeld, 1990; Coligan et al., 2001) have been developed in which the expressed product is a fusion protein containing an N-terminal polypeptide that simplifies purification. Such "tags" can be subsequently removed using a specific protease. A further advantage is that the expression level is dictated mainly by the transcription and translation signals for the fusion portion of the protein, which are optimized. Tags used include proteins and polypeptides for which there is a specific antibody, binding proteins that will interact with columns containing a specific ligand, polyhistidine tags with affinity to immobilized metal columns, sequences that result in

biotinylation by the host and enable purification on an avidin column, and sequences that confer insolubility under specified conditions.

Unstable proteins may be modified by the molecular biological technique of site-directed mutagenesis to remove the site of instability—for instance, an oxidizable cysteine. Such techniques are appropriate for commercial production of proteins, but may of course alter natural functioning parameters. Increased thermostability can be one modification, although it is not easy to predict mutations that will improve that parameter. Thermostable proteins originating from thermophilic bacteria do not need structural modification and, if expressed in large amounts, can be purified satisfactorily in one step by simply heat-treating the extract at 70°C for 30 min, which denatures virtually all the host proteins (e.g., see Oka et al., 1989).

The host bacteria used for production of recombinant proteins are usually *E. coli*, or *Bacillus subtilis*; they may express proteins at 1% to over 50% of the cellular protein, depending on such variables as the source, promoter structure, and vector type. Generally the proteins are expressed intracellularly, but leader sequences for excretion may be included. In the latter case, the protein is generally excreted into the periplasmic space, which limits the amount that can be produced. Excretion from gram-positive species such as *B. subtilis* sends the product into the culture medium, with little feedback limitation on total expression level.

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Overview of Conventional Chromatography

The key to success in purification of any protein is developing the right separation strategy. This involves selecting and correctly applying a combination of separation techniques, chosen on the basis of information about the target protein and contaminants. There is no single separation technique and no single purification scheme that will allow successful purification of all types of proteins. However, there is a single approach to developing a successful purification strategy.

Three basic steps must be completed before beginning to develop a purification strategy. First, the amount and purity of the target protein (the protein to be purified) required for its intended use must be determined. Second, all available physical and chemical information concerning potential source materials, the nature of the target protein itself, and major contaminants must be gathered. Third, the best source material from which to purify the target protein must be determined. These three steps are mutually interdependent—what is learned in one will have an impact on the others. The more that is known before beginning development of a purification strategy, the more successful that strategy will be.

YIELD AND PURITY OF TARGET PROTEIN

How much protein must be purified? How pure must the protein be? These two questions establish the criteria for selecting specific chromatographic techniques and for judging the efficacy of any individual purification step or overall purification procedure. The answers to these simple questions depend upon the nature of the source material from which the target protein will be purified, the intended use for the purified protein, and the methods available to assess the purity of the target protein.

Amount of Protein Required

The amount of protein required from a purification process depends primarily on the intended use for the target protein. However, the amount of protein that will be used during the purification process to develop methods and assess purity must also be considered. It is necessary only to estimate whether analytical (≤1 mg), preparative (≤1 g), pilot (≤ 1 kg), or process (>1 kg) amounts of target protein will be needed. This is often referred to as the scale of operation. When the source material is very expensive, unstable, or difficult to obtain, it is necessary to develop and optimize the purification strategy on a small scale first, then scale up. Conversely, if multiple samples are to be processed, as for analytical applications, it may be desirable to develop the purification process at a larger scale, then scale down. The amount of target protein to be purified may also be determined by limited availability of source material.

Source Material

Once the scale of operation has been determined, the amount of source material needed to begin the procedure must be clearly defined. This obviously depends on the concentration of target protein in the source material, which can vary greatly. Certain proteins and peptides are present in natural sources only in very minute quantities. Purification of even a microgram of pure material may require many kilograms of tissue, or many liters of fermentation broth if microorganisms are the source. On the other hand, some recombinant proteins are expressed at such high levels in a host that the protein may be present in milligram per milliliter or higher concentrations. Source materials containing very low or very high concentrations of target protein each present unique problems that must be addressed in selecting a purification strategy.

Yield of Purification Procedure

The next major consideration in deciding how much protein to purify is what yield is necessary at each step of the purification procedure to ensure that there will be enough pure target protein at the end, taking into account the availability of source material. If a 75% yield of target protein is obtained at each of four steps in a purification procedure, less than one-third of the desired protein will remain at the end of the procedure (Fig. B4.2.1). It is obviously advantageous to use as few steps as possible in a purification process while maintaining as high a yield as possible at each step.

The yield from any purification step may be reported as total protein, percentage of total activity, or change in specific activity. The yield information from progressive purification steps is used to construct a purification table (e.g., see Table B4.2.1).

In Table B4.2.1, the yield is reported as percent of total activity remaining from the

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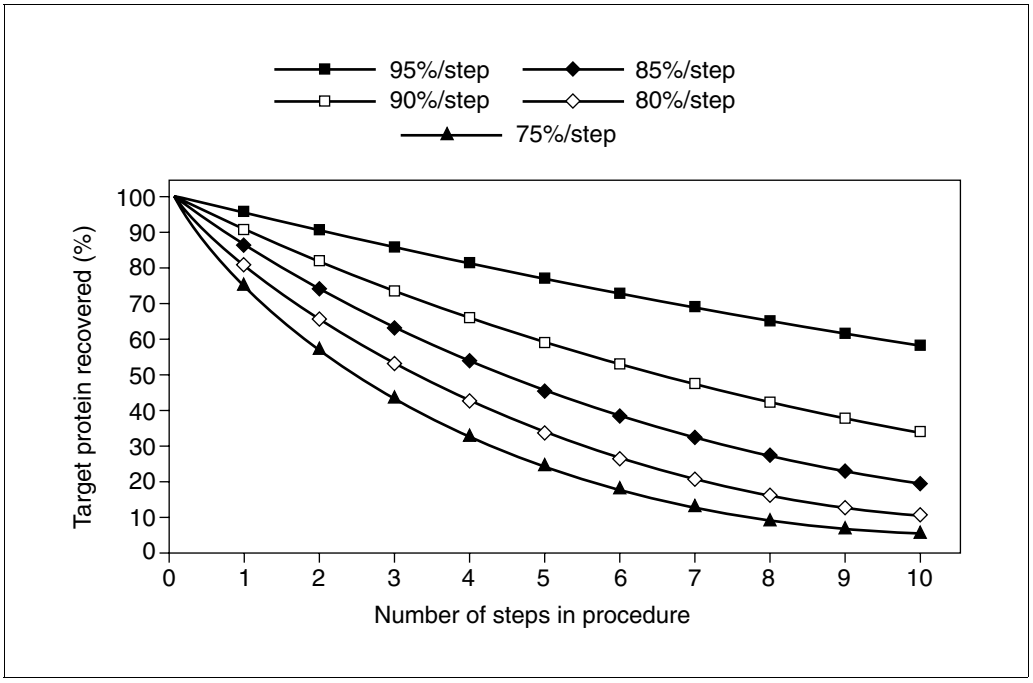

Figure B4.2.1 Theoretical yields from multistep protein purifications. Each curve represents a multistep process with a given percentage yield per step. The percentage of the original quantity of target protein remaining at the end of purification is plotted against the number of purification steps.

crude homogenate; the step yield is the percent activity relative to the previous step; and the purification factor reflects the change in specific activity relative to the crude homogenate. It is not unusual to have yields >100% based on activity (e.g., the yield after anion-exchange chromatography in Table B4.2.1). This is most often due to the removal of inhibitors or contaminants that interfere with the activity assay.

Assessment of Protein Purity

Assessing protein purity is often the most time-consuming and labor-intensive step in the purification process. It is generally desirable to limit the number of steps in a purification process simply to reduce the number of assays that must be performed to assess purity at each step. Protein purity is judged from a structural and/or functional perspective, depending on the nature of the target protein and its intended use. If the intention is to examine the function of the target molecule, the activity of the target protein must be maintained during the purification process. A functional assessment of the purity of the target protein will thus be required at each step. If the intention is to examine the structure of the target molecule, structure must be maintained, but function may or may not have to be preserved. A structural assessment of purity will, however, be required in this case. If structural/functional relationships involving the target molecule are to be examined, assessment of both structural and functional purity will be required.

Methods for assessing protein purity must quantitate the amount of target protein relative to the amount of contaminant(s) in the sample. This requires two separate analytical methods: one for the target protein and one for the total protein including contaminants. For example, with enzymatic proteins, assessment of functional purity typically relies on kinetic assays in which the amount of substrate consumed or product produced per unit time is proportional to the amount of enzyme present. The total activity of the sample is calculated and compared to the total amount of protein to give the specific activity of the sample. Methods for determining total protein are discussed in Chapter B1. An increase in specific activity at a purification step reflects a loss of contaminant proteins.

Functional purity of structural proteins, denatured proteins (i.e., proteins that have lost their function during the purification procedure), and proteins for which a functional assay does not exist must usually be assessed by measuring structural attribute(s)—e.g., molecular weight, pI, presence of a metal ion or co-factor, or presence of an antibody binding

Table B4.2.1 Purification Table for an Arbitrary Enzyme[a]

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (% total activity)	Step yield[b]	Purification factor[c]
Crude homogenate	200	50,000	0.004	100	—	1.00
12,000 × *g* supernatant	180	35,000	0.005	90	90	1.25
$(NH_4)_2SO_4$ fraction (20%-50%)	150	25,000	0.006	75	83	1.50
HIC	125	2500	0.05	62.5	83	12.50
AEX	208	29.3	7.1	104	166	1775
Gel filtration	148	5.9	25.1	74	71	6275

[a]Abbreviations: HIC, hydrophobic-interaction chromatography; AEX, anion-exchange chromatography.

[b]Percentage activity relative to previous step.

[c]Increase in specific activity.

site. As in the case of functional purity, an increase in the amount of target protein relative to total protein reflects an increase in purity.

STEPS IN A PURIFICATION STRATEGY

A purification strategy for any protein at any scale of operation can be broadly divided into three sequential stages—capture, intermediate purification, and polishing. Each stage represents a set of specific problems that may be encountered during a purification process. The nature of the sample and the scale of operation will dictate what equipment and methodology are appropriate to solve the problem. Many of the procedures discussed here can be found in Coligan et al. (2001).

Capture Stage

The capture stage is the initial purification of the target protein from the source material. The goal of capture is to concentrate the target protein while removing as much of the major contaminant(s) as possible. Although a selective batch precipitation step can be used successfully at this point, this unit will focus solely on chromatographic techniques for purification. An adsorptive chromatographic technique is often used in the capture stage. Ion-exchange chromatography and hydrophobic-interaction chromatography (HIC) are generally the best chromatographic techniques for capture at any scale of operation. Certain affinity techniques are also effective, including lectin affinity chromatography, dye affinity chromatography, immunoaffinity chromatography, and metal-chelate affinity chromatography (MCAC). Affinity chromatography is very useful for capture on a small scale (e.g., <1 g). Larger-scale capture using affinity-based methods is generally avoided because of the cost of the chromatographic media and reagents (especially for immunoaffinity chromatography).

At the capture stage the sample is very crude. It may be turbid from the presence of cellular debris or viscous from the presence of DNA, and will typically represent the largest volume to be processed during the purification procedure. Water is often the major contaminant, especially in samples from cell-culture fluids or fermentation broth. The sample may also contain pigments, particulates, and lipophilic materials that can easily clog chromatography columns or bind nonspecifically to certain media. Once such contaminants are introduced into a chromatography system, they can be very difficult to remove. The sample may also contain proteinases or other agents that can potentially destroy or denature the target protein.

Ion-exchange or HIC media suitable for capture must allow high throughput of dilute, turbid, or viscous samples. This is achieved by using bead diameters of 40 to 300 µm. Columns packed with such relatively large beads can usually be operated at lower pressures and higher flow rates, and are less prone to fouling or clogging from particulates in the sample. Higher flow rates reduce the time required to handle large volumes, which in turn reduces the potential for product loss from degradation or denaturation.

When the volume of crude sample is very large (or larger than can be reasonably handled with available pumps or columns), batch techniques can be used at the capture stage. In batch adsorption of the target protein by ion ex-

change, HIC, or affinity chromatography, the chromatography medium is mixed directly with the sample in a suitable vessel. When the target protein is adsorbed, the medium is separated from the sample by filtration, centrifugation, or settling and decantation. The target protein is then eluted from the medium and collected. For both column and batch techniques, elution of the target protein at the capture stage is often achieved using simple step gradients, but continuous gradients may be used to achieve higher resolution with column techniques.

Intermediate Purification Stage

The next stage in the purification process is intermediate purification. Once the solution containing the target protein has been clarified and concentrated, only small amounts of other types of biomolecules (e.g., lipids and nucleic acids) should be present. The target protein and remaining protein contaminants have a functional or structural attribute in common, whose nature depends on the technique that was used at the capture stage. If ion exchange was used, the contaminants and target protein will be similar in charge characteristics; if HIC was used, similar in hydrophobicity; if affinity methods were used, similar in a ligand- or antibody-binding site; and if ultrafiltration or gel filtration was used, similar in size.

Because the contaminants have a physicochemical similarity to the target protein, it is best to exploit a different attribute of the target protein or contaminant during the intermediate purification stage than was used during the capture stage. Use of the same chromatographic technique for both capture and intermediate purification is not recommended. The combination of ion exchange and HIC is very effective in most purification strategies. Proteins are eluted from HIC media at low ionic strength, which is ideal for direct application of the sample to an ion-exchange column, where binding occurs at low ionic strength. Conversely, proteins are eluted from ion-exchange media with increasing ionic strength, which is ideal for subsequent direct application of the sample to an HIC column, where binding occurs at high ionic strength. Cation and anion exchange in combination can also be very effective. The reduced sample volume in the intermediate purification stage also allows use of negative adsorption chromatography, which is designed to bind contaminants to the column, allowing the target protein to pass through.

Chromatography techniques used in intermediate purification should resolve the target protein from contaminants on the basis of small differences in a single physicochemical attribute. High resolution and high yield are the primary goals of intermediate purification. To achieve these goals, a chromatography medium of smaller bead size than that used at the capture stage should be selected. The advantage of smaller bead diameters is increased resolution resulting from decreased peak width. However, smaller bead diameters typically require higher operating pressures. To further enhance resolution during intermediate purification, elution of the target protein is often done using a linear gradient.

Polishing Stage

Polishing is the final stage in the purification process. The goal of polishing is to remove structural and functional variants of the target protein, as well as any trace contaminants. After polishing, the target protein should be in a suitable form for its intended use. The contaminants and structural variants that must be removed during polishing share very similar physicochemical attributes with the target protein. Chromatographic techniques employed in the polishing stage should exploit a different physicochemical attribute of the target protein than those used for capture and intermediate purification. The similarity of contaminants to target protein heightens the need for high-resolution techniques during this stage. Chromatofocusing, gel filtration, reversed-phase chromatography, and affinity-based techniques are all useful in this stage. Reversed-phase chromatography is very effective for resolving structural variants of a target protein, but generally leads to loss of activity of the target protein because of the organic solvents employed. Adsorptive techniques such as chromatofocusing and affinity chromatography often require special eluants to recover the target protein, which may have to be removed before the purified target protein can be used. Gel filtration does not generally require any special eluants, and is often the best technique for polishing.

Optimizing Purification

Throughout the purification process, the concentration of target protein increases with respect to the concentration of contaminants. In addition, the contaminants to be removed at each stage are increasingly similar to the target protein. This increases the need for higher resolution and better yields at each progressive step.

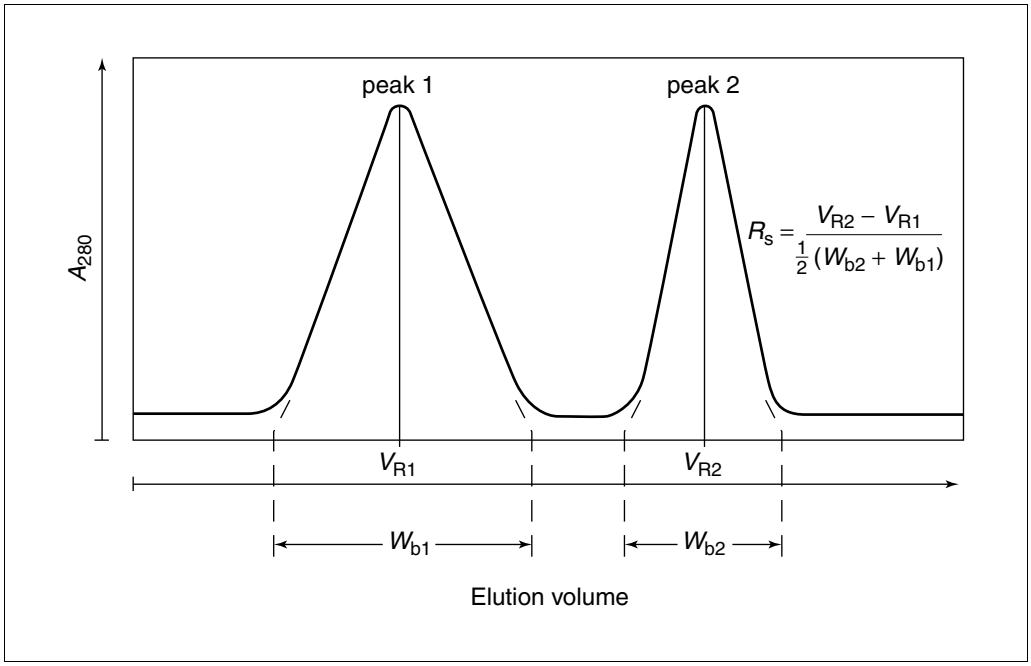

Figure B4.2.2 Hypothetical chromatogram illustrating calculation of elution volumes and peak widths for two peaks. Total elution volume is plotted against absorbance at 280 nm. V_{R1} and V_{R2} are the elution volumes (at the peak maxima) of peaks 1 and 2, respectively; W_{b1} and W_{b2} are the peak widths (at base) of peaks 1 and 2, respectively. Peak widths at base represent the segment of the baseline intercepted by the tangents drawn to the inflection points of the peak. Resolution (R_s) is defined as the distance between the elution volumes at peak maxima divided by the average of the peak widths, according to Equation B4.2.1 (see Resolution).

It is desirable to examine the efficacy of several techniques at each stage of the procedure before deciding on a specific one to use. Once a specific chromatographic technique has been chosen, the methodology for that step should be optimized: first to achieve maximum resolution, then maximum capacity and yield, and finally maximum speed. This approach will result in a fast, efficient, economical, and robust purification strategy.

PARAMETERS AFFECTING CHROMATOGRAPHIC RESOLUTION

Resolution

Resolution is a measure of the relative separation achieved between two chromatographically distinct materials, and maximum resolution is the primary goal of any purification step. This discussion covers the main theoretical parameters that affect ability to resolve components of a protein sample into chromatographically distinct forms, thereby providing a basis on which to judge chromatographic results. The main parameters affecting resolution are selectivity, efficiency, and capacity. More detailed discussions are found in Giddings and Keller (1965) and Janson and Ryden (1989).

The result of any chromatographic separation is often expressed as the resolution between the zones or peaks containing the chromatographically distinct sample components. The resolution (R_s) of two peaks obtained in a chromatography step may be determined from the chromatogram shown in Figure B4.2.2 according to Equation B4.2.1, where V_{R2} is the elution volume of peak 2, V_{R1} is the elution volume of peak 1, W_{b1} is the width of peak 1, and W_{b2} is the width of peak 2. In cases where a constant eluant flow is maintained and fractions are collected at regular time intervals, V_{R1}, V_{R2}, W_{b2}, and W_{b1} may be expressed in units of time rather than volume. It is necessary that the same units (time or volume) be used to express all terms in the equation to give a dimensionless value to R_s.

$$R_s = \frac{V_{R2} - V_{R1}}{\frac{1}{2}(W_{b2} + W_{b1})}$$

Equation B4.2.1

Resolution values can be used to determine if further optimization of a chromatographic procedure is required, as they provide a numerical indication of whether two peaks have been completely resolved. Figure B4.2.3 compares two separation results: one with completely resolved peaks and the other with incompletely resolved peaks. It should be kept in mind that a completely resolved peak is not necessarily a pure substance. A single peak frequently represents multiple substances that are not resolved by the particular chromatographic technique used.

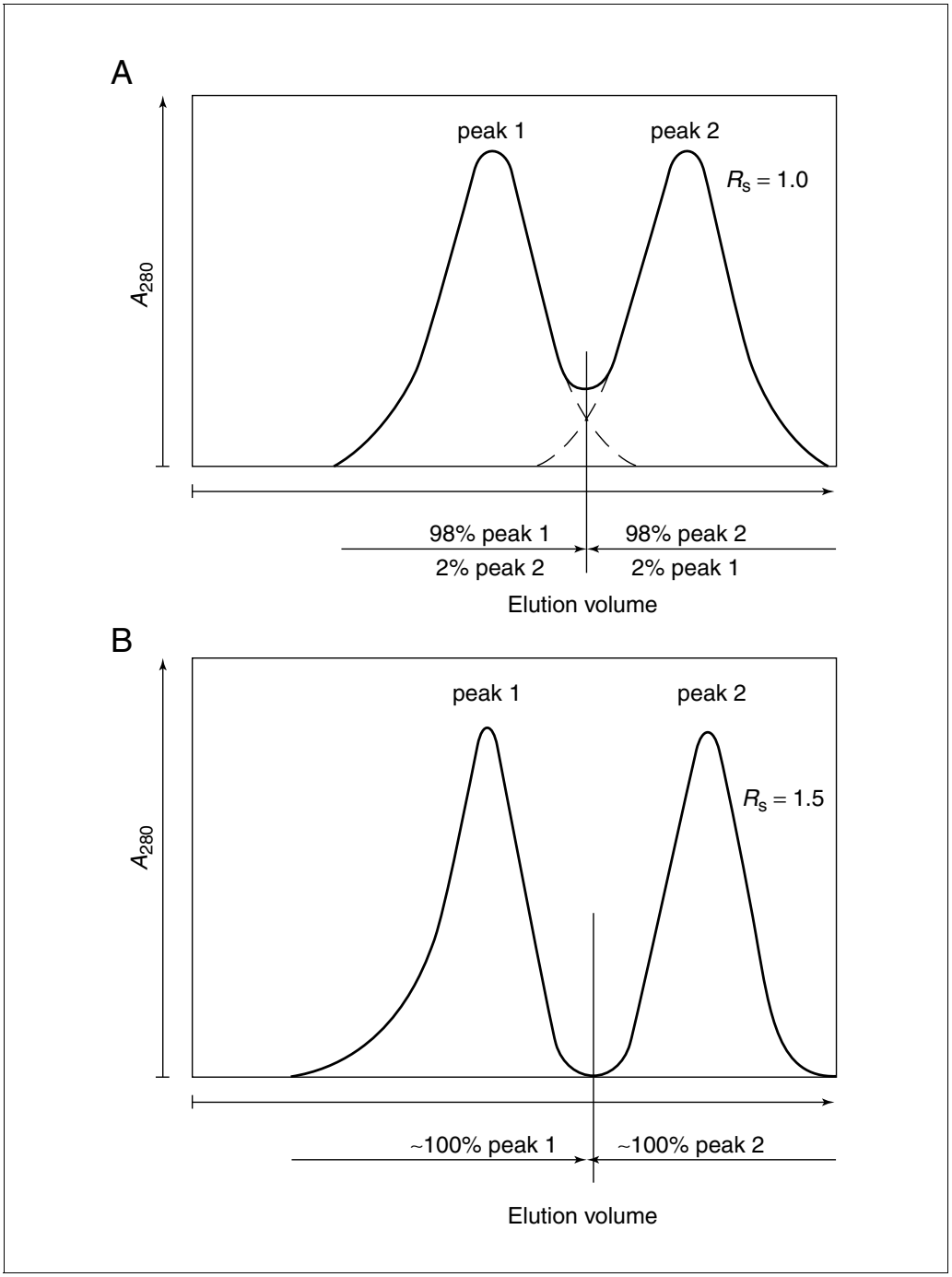

Figure B4.2.3 Separation results with different resolutions. (**A**) When R_s = 1.0, 98% purity has been achieved—i.e., 98% of the fraction collected at peak 1 consists of a single protein with the remaining 2% being contamination from the protein collected at peak 2; conversely, 98% of the fraction collected at peak 2 consists of a single protein with the remaining 2% being contamination from peak 1. (**B**) Complete (baseline) resolution requires that R_s >1.5. At this value, the peaks are completely separated from each other (i.e., the purity of each peak is >99.9%).

The resolution achievable in any chromatographic system is proportional to the capacity, efficiency, and selectivity of the system. Each of these factors must be considered and controlled to achieve success. The theoretical expression for resolution is Equation B4.2.2, where k is the average capacity factor for the two peaks, N is the efficiency factor for the system, and α is the selectivity factor of the medium.

$$R_s = \frac{1}{4}\left(\frac{k}{1+k}\right)\left(N^{-2}\right)\left(\frac{\alpha-1}{\alpha}\right)$$

Equation B4.2.2

Capacity

The capacity or retention factor (k) is a measure of retention of a sample component. It should not be confused with the loading capacity of a column, which is expressed as milligrams of sample bound per milliliter of gel and represented by the area under a peak. The capacity factor may be calculated for any individual peak in a chromatogram. For example, the capacity factor for peak 2 in Figure B4.2.4 is derived from Equation B4.2.3, where V_{R2} is the elution volume of peak 2 and V_M is the volume of the mobile phase (i.e., the total bed volume).

$$k = \frac{V_{R2} - V_M}{V_M}$$

Equation B4.2.3

In Equation B4.2.2 for R_s, k is the average of k_1 (capacity factor for peak 1) and k_2 (capacity factor for peak 2).

Adsorption techniques such as ion exchange, HIC, chromatofocusing, reversed-phase chromatography, and affinity chromatography can have high capacity factors because experimental conditions can be manipulated so that the elution volume for a peak can exceed the total bed volume (V_M as is the case with peaks 2 and 3 in Fig. B4.2.4.) However, in gel filtration, which is a nonadsorptive technique, all peaks must elute within the volume V_M–V_0, as is the case with peak 1 in Fig. B4.2.4.

Efficiency

The efficiency factor (N) is a measure of zone broadening (peak width) occurring on a column. It can be calculated for any given peak from Equation B4.2.4, where V_R is the elution volume (i.e., the total volume of eluant that has passed through the column at peak maximum) and W_h is the peak width at half the peak height.

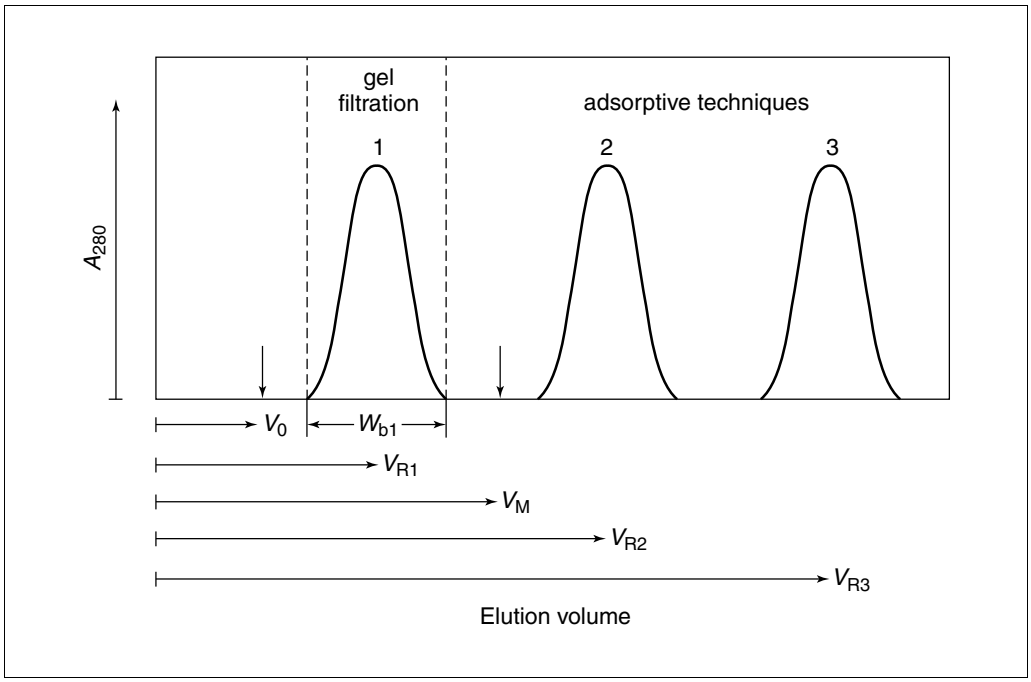

Figure B4.2.4 Hypothetical chromatogram. V_0 = void volume; V_{R1} = elution volume for peak 1; V_{R2} = elution volume for peak 2; V_{R3} = elution volume for peak 3; V_M = volume of mobile phase; W_{b1} = peak width for peak 1.

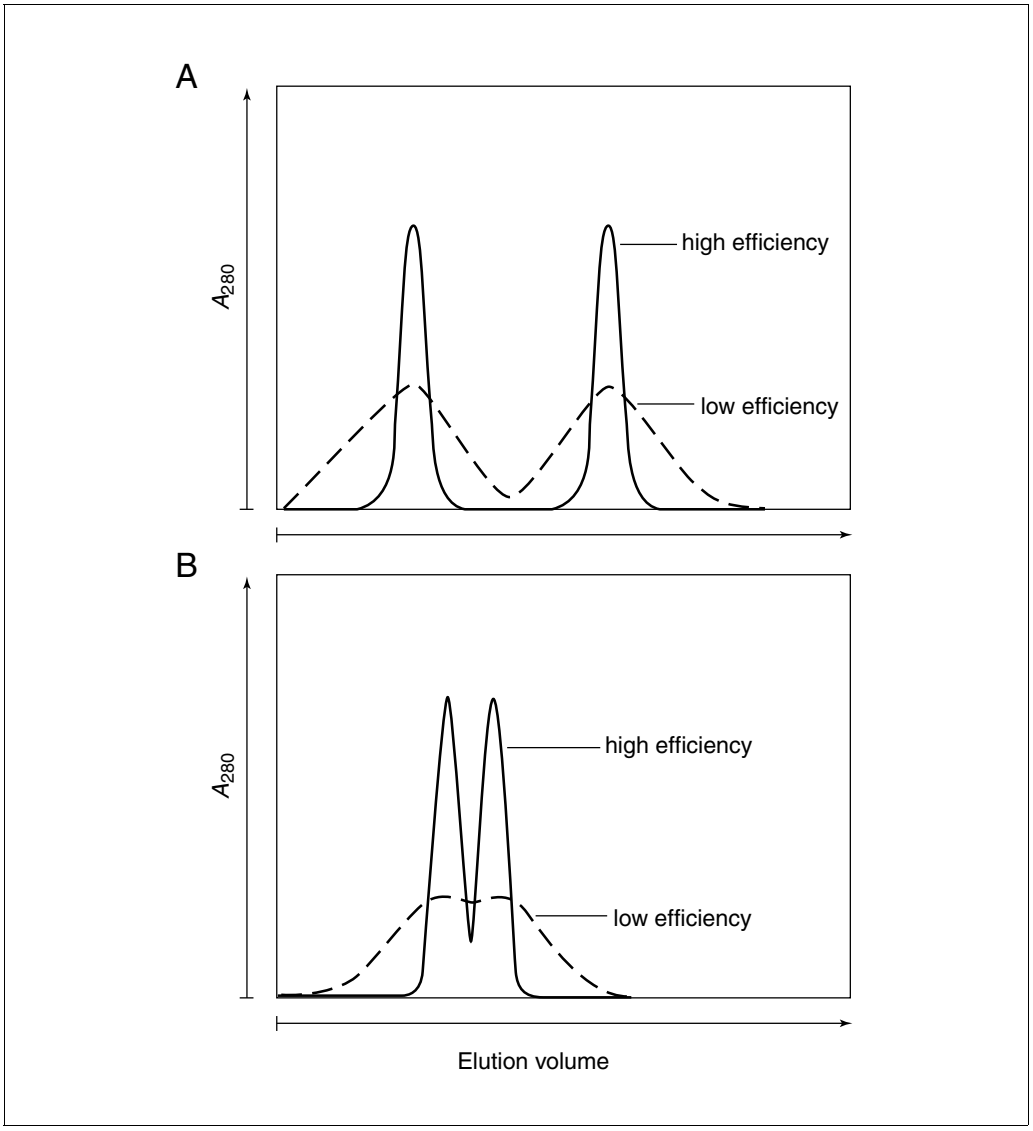

Figure B4.2.5 Effect of selectivity and efficiency on resolution. (**A**) Chromatogram obtained using experimental technique that provides good selectivity. Where conditions are chosen to provide high efficiency, two narrow and distinct peaks are obtained (high resolution). When the conditions provide only low efficiency, the peaks are broadened and overlap more (lower resolution). (**B**) Chromatogram obtained using experimental technique that provides bad selectivity. Even when high-efficiency conditions are used, there is lower resolution than in (A), and the resolution deteriorates further with low-efficiency conditions.

$$N = 5.54\left(\frac{V_R}{W_h}\right)^2$$

Equation B4.2.4

Efficiency (N) may be expressed as the number of theoretical plates for the column under specific experimental conditions. Efficiency is also frequently defined as the number of plates per meter of chromatographic bed, or in terms of H, the height equivalent to a theoretical plate. H is simply the column length (L) divided by the efficiency factor (N); i.e., $H = L/N$.

The main cause of zone broadening (i.e., loss of efficiency) in a chromatographic bed is diffusion. Diffusion perpendicular to the flow is restricted by the walls of the column. Therefore, longitudinal diffusion is the primary factor contributing to zone broadening. While a protein is adsorbed to the medium, little or no diffusion takes place, but once the protein is unbound, diffusion begins. The amount of diffusion that occurs is proportional to the time required for the material to emerge from the

system. Loss of efficiency resulting from diffusion is minimized if the distances available for diffusion in the mobile phase, gel beads, and system are minimized. In practice, efficiency increases with increasing uniformity in particle size and with decreasing bead size. Good experimental technique is required for high efficiency. Unevenly packed chromatography beds and trapped air will lead to channeling, zone broadening, and consequent loss of resolution. Loss of efficiency also stems from system effects such as dead volumes in the system, poor mixing during gradient formation, and pulsations in flow.

Selectivity

Selectivity of a chromatographic medium defines the ability of that medium to separate peaks (i.e., it is a measure of the distance between two peaks in a chromatogram; see Fig. B4.2.5). The selectivity factor (α) can be calculated from a chromatogram using Equation B4.2.5, where k_2 is the capacity factor for the second peak (see Capacity), k_1 is the capacity factor for the first peak, V_{R2} is the elution volume of the second peak, V_{R1} is the elution volume of the first peak (see Fig. B4.2.2), and V_M is the volume of the mobile phase.

$$\alpha = \frac{k_2}{k_1} = \frac{V_{R2} - V_M}{V_{R1} - V_M} \approx \frac{V_{R2}}{V_{R1}}$$

Equation B4.2.5

Selectivity is more important than efficiency (N) in determining resolution because R_s is directly proportional to selectivity, but is proportional only to the square root of efficiency (see Fig. B4.2.5 along with Equation B4.2.1 and Equation B4.2.2. Hence, a four-fold increase in efficiency is required to double resolution, as compared with a two-fold increase in selectivity. In practice, selectivity depends partly on the chromatographic technique employed but can usually be controlled by manipulating experimental conditions, such as the pH and ionic strength of the mobile phase. Because this can be done easily and predictably, selectivity is the factor that is exploited to achieve maximum resolution in column chromatography rather than efficiency, which is fixed by the particle size and uniformity of the medium selected.

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Measurement of Functional Properties: Overview of Protein Functionality Testing

INTRODUCTION

Functionality is a term used to describe those characteristics of a food that have been correlated to quality attributes identified by the human senses. Proteins play important roles in the functional properties of many foods, and thus contribute to the quality and sensory attributes of many food products. Protein functional properties have traditionally been defined as physical or chemical properties of proteins that affect their behavior in food systems during preparation, processing, storage, and consumption. Protein functional properties can be classified into three broad categories: (1) hydration properties, such as solubility or water retention; (2) surface properties, such as emulsification and foaming; and (3) protein-protein interactions, such as gelation. Functional properties are often the result of several physical or chemical reactions occurring simultaneously or sequentially in a food during processing and are not easily measured using a single physical or chemical test. One protein in a food may be primarily responsible for the desired functional properties, or a group of proteins may be involved.

To further complicate the situation, often more than one functional property is required in a particular food system. Thus, a protein or a group of proteins must be multifunctional. Egg proteins are required to form a foam and also to form a gel, as well as to emulsify lipid and hold water, in order to form a custard with the desired quality attributes. Often, the requirement for a particular functionality will change during preparation and processing. For example, muscle proteins hold water, enhance viscosity, and may emulsify lipid in raw comminuted meat products, but must form gels with high water-holding ability upon heating to achieve the desired textural properties that consumers expect and to deliver the processing yields required by processors.

The functionality of a protein is dictated by the molecular properties of the protein as modified by processing treatments, environmental factors, and interactions with other components. Environmental conditions, such as pH, ionic strength, type of salts, moisture content, and oxidation-reduction potential, may alter the functional properties of a protein in a food. Protein functional properties are also influenced by unit operations during processing such as heating, drying, shearing, pressure, and freezing. Protein functional properties often change during storage due to chemical or physical reactions such as protein aggregation, denaturation, enzyme activity, lipid oxidation, ice-crystal damage, or other factors. Similarly, interactions with other macromolecules—such as carbohydrates, lipids, or even other proteins—during processing and storage can change the functional properties of a protein.

Foods are highly complex systems comprising multiple ingredients. It is often difficult to isolate and understand the role of a particular protein in a food system due to the myriad of interactions that occur between food components during preparation and processing. For this reason, scientists often elect to devise model systems to elucidate the functional properties of proteins in a simple system under carefully defined and controlled conditions. This approach may eliminate many confusing variables and simplify data interpretation. However, results of model system tests must often be interpreted carefully, as results obtained in a simplified system may not translate accurately to a real food system. A wide variety of model system tests are used, but many do not accurately predict the function of a protein in a real food system. Model system tests are often chosen at random, sometimes based solely on the equipment available in a particular laboratory. Conditions (such as energy input, pH, ionic environment, and heating rate) used in model systems are often different from those used during actual processing operations. Additionally, methods are often modified slightly by each user, such that results obtained in one laboratory may not relate to results obtained in another.

SELECTING A FUNCTIONAL TEST

The literature is replete with methods to measure protein functional properties. For example, Kneifel et al. (1991) listed about 70 published methods to measure the water-holding ability of dairy proteins. Table B5.1.1, Table B5.1.2, and Table B5.1.3 list some common tests used to evaluate the functional properties of proteins. The methods in these tables should serve as examples only. Selection of the proper test to evaluate a particular functional property is difficult. A functional property test must meet the needs of the user and answer the question(s)

Contributed by Denise M. Smith

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at hand. Any method should be tested before use to make sure results will correlate with the real food system under study. Mangino (1989) stated that the method of functional property evaluation many have a greater effect on the results than the actual variables under study. Some methods are highly standardized procedures, whereas others are specific to a particular laboratory. Some methods are highly empirical, others are more fundamental. No single test will work for all applications.

Many standardized functional property tests have been published. Standardized methods are available from many organizations, including the American Association of Cereal Chemists, American Oil Chemists Society, and International Dairy Federation. Generally, these methods have been tested in collaborative studies by several laboratories and found to be repeatable. Therefore the use of standardized methods may facilitate comparison of results between laboratories. Even though a method has been standardized, there is no guarantee that the test results will relate to protein functionality in a particular product or product application. Another problem is that most of the standardized tests are empirical. If any step in the procedure is changed or equipment is substituted, comparisons between laboratories may not be possible.

The following paragraphs describe a number of factors that should be considered when selecting a functional property test.

Terminology

Functional property terminology is not well defined and is not universally agreed upon. Often, the same term is used to describe a variety of methods measuring different properties. For example, terms such as water hydration capacity, water absorption, water binding, and water-holding capacity are all used interchangeably to describe water bound or retained by a protein. It is therefore necessary to carefully define the terms under study and make sure the methods selected measure the desired parameters.

Complexity of the Model System

Before selecting a method to measure a specific aspect of protein functionality, one must decide on the complexity of the testing matrix. Researchers have used a single purified protein, a crude extract of proteins, a prototype food product, or an actual product to study protein functionality. For meat studies, formulated meat systems, ground muscle, myofibrillar proteins, salt-soluble proteins, actomyosin, myosin, or even subfragments of myosin have been used to study the functional properties of muscle proteins. For infant formulas, prototype formulas, milk proteins, whey proteins, casein, or individual milk proteins such as α-casein or α-lactoglobulin have been used as the testing matrix. The presence of carbohydrates and lipids in a formulation may affect protein functionality, and thus it may be important to include those macromolecules in the model system. In general, the more complex the system, the more likely are the results to correlate with the real food system. However, confounding factors caused by interactions of ingredients may lead to inaccurate conclusions and an inability to elucidate a fundamental understanding of the underlying reactions.

It is often best to begin with purified proteins when studying the relationships between protein structure and function at the molecular level. The presence of multiple proteins often complicates data interpretation, as it is not clear if effects are due to protein interactions, variations in the ratio of proteins, or to other factors. In these studies it is advisable to select tests based on a fundamental physical or chemical property, since results are less likely to vary with the test conditions or instrumentation used. Unfortunately, it becomes less likely that the property under study will relate directly to function in a food system when such simplified (often dilute) systems are used. Something as seemingly insignificant as protein concentration in the model system can have a large influence on the results obtained. Also, the relative importance or contribution of a functional property to a complex food system can be misinterpreted in a purified model system.

Environmental Conditions

Protein function is dependent on environmental conditions such as pH, ionic strength, and ionic composition. The environmental conditions used in many published tests of protein functionality differ greatly from those in the food under study. The farther the environmental conditions diverge from those of the real food, the less likely are the functional test results to predict performance. It is best to select environmental conditions that closely mimic those found in the real product under study.

Equipment Selection

It is best to select processing conditions and use equipment that most closely mimic those actually used during processing of the food. Many functional property tests are empirical and are

method- and equipment-dependent. Consequently, any change in processing equipment may affect the results and not allow valid comparisons between laboratory tests and what actually occurs in the processing plant environment.

Tests of protein emulsification can be used to illustrate this problem. One important factor determining the properties of an emulsion is the method of emulsion formation. It is known that energy input during emulsification has a large effect on droplet size, which subsequently influences emulsion stability. Published procedures describe the use of various homogenizers, blenders, or even shaking to form an emulsion, with no consideration given to energy input or the method of emulsion formation used in the processing plant. Absolute numbers are rarely the same when properties of emulsions made using different equipment are compared; however, in some cases the rank order of treatments may be the same (Mangino, 1989).

CONCLUSIONS

Model systems tests have played an important role leading to our understanding of protein functionality in food systems. There is no single test that is applicable for all food systems. Careful consideration must be given when designing a model system and selecting a specific protein functionality test for a particular application. Comparison of results from different methods and from other laboratories must be made with caution. Similarly, extrapolation of results from model systems to real food products must also be carefully interpreted.

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Measurement of Protein Hydrophobicity

It is generally accepted that hydrophobicity, especially surface or effective hydrophobicity, plays an important role in functionality of food proteins. Therefore, measurement of the hydrophobicity is essential for understanding protein fucntionality. The most popular method for measuring the surface hydrophobicity of proteins is probe spectrofluorometry, probably because of the simplicity and rapidity of analysis. Many hydrophobic probes have been used for food proteins, such as 1-anilinonaphthalene-8-sulfonic acid and *cis*-parinaric acid (ANS and CPA, respectively; see Basic Protocol 1), 1,6-diphenyl-1,3,5-hexatriene (DPH; see Alternate Protocol 1), and 6-propionyl-2-dimethylaminonaphthalene (prodan; see Alternate Protocol 2). A drawback of this fluorescent probe approach is the need for a spectrofluorometer, which is more sensitive and specific than a spectrophotometer, and thus more expensive. Alternatively, the detergent binding capacity is occasionally measured (see Basic Protocol 2). Hydrophobic-interaction chromatography (see Basic Protocol 3) has been used extensively, but interpretation of the results obtained is controversial because of the possible surface denaturation of sample proteins. Contact angle determination (see Basic Protocol 4) is a unique physical measurement, but is seldom used for proteins. Hydrophobic partition (see Basic Protocol 5) is a method that is more frequently used; it is theoretically interesting, but is also more time consuming.

PROBE SPECTROFLUOROMETRY USING ANS OR CPA

BASIC PROTOCOL 1

This method is the most popular and widely used because it is quick and simple. Samples are measured in the presence of fluorescent probes whose fluorescence increases under hydrophobic circumstances. In general, fluorescent spectra and quantum yields are highly dependent on the environment, especially solvent polarity (Stryer, 1965). The quantum yield is the proportion of the total photon emission in the entire fluorescence spectral profile. The quantum yield of ANS in water is 0.004, which increases to 0.37 and 0.63 in ethanol and *n*-octanol, respectively. In a similar fashion, dissolving a protein in water increases its quantum yield depending on solvent-accessible (i.e., surface) hydrophobic sites of the proteins. Apomyoglobin increased this value to 0.98 through its noncovalent binding with ANS. The dipolar excited state of a fluorophor (probe) interacts with a polar solvent so as to orient the solvent dipoles, thereby dissipating the excited energy, whereas the solvent shell in nonpolar solvent is less perturbed. A low-energy photon is emitted by the fluorophor in a polar solvent, as some of the solvation energy of the excited state is lost on photon emission; this phenomenon occurs to a lesser extent in nonpolar solvents. Unlike ANS, which is a synthetic chemical compound, CPA is the naturally occurring polyunsaturated fatty acid, and is therefore useful in simulating lipid-protein interactions, which are frequently seen in food systems.

Materials

Protein solution to be tested
0.01 M phosphate buffer, pH 5.5 to 7.4 (*APPENDIX 2A*), containing 0 to 0.6 M NaCl
Fluorescent probe (Molecular Probes), select one:
- 8.0 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer, pH 7.0
- 36 mM *cis*-parinaric acid (CPA) in absolute ethanol containing 3.6 mM butylated hydroxytoluene (BHT)

Methanol or decane
Spectrofluorometer with cuvettes (all sides quartz)

Contributed by Shuryo Nakai
Current Protocols in Food Analytical Chemistry (2003) B5.2.1-B5.2.13

1. Prepare serial dilutions of the protein solution ranging from 0.005% to 0.03% (w/v) in 0.01 M phosphate buffer, pH 5.5 to 7.4, containing 0 to 0.6 M NaCl.

   Specific buffer conditions (pH and salt) are selected for the environment of the food sample to be simulated. Generally, pH 7.0 and 0 NaCl are appropriate.

2. Prepare duplicate 2-ml aliquots of each protein concentration.

3. Add 10 µl ANS or CPA fluorescent probe to one tube at each concentration, and add 10 µl of the solvent used to prepare the probe solution to the other tube as a control (blank).

4. Standardize the spectrofluorometer by adjusting the reading for equivalent concentrations of ANS in methanol or CPA in decane to an arbitrary value, such as 70% as the full scale.

   These conditions should be the same as for samples and blanks.

5. Measure the relative fluorescence intensity (RFI) of each sample and control with a spectrofluorometer at excitation and emission wavelengths (λ_{ex}, λ_{em}), respectively, of 390 and 470 nm (for ANS) or 325 and 420 nm (for CPA).

6. Compute the net RFI for each sample concentration by subtracting the corresponding blank.

7. Plot net RFI versus protein concentration (%), and then find the slope (S_0, a hydrophobic index) of the line by linear regression analysis (or the initial slope at the origin of quadratic fitting for a more precise measurement; as seen in Figure B5.2.1, curve 1).

   Because RFI is a relative value, S_0 is also relative. Thus, in practice, relative S_0 is computed against the corresponding value of a standard protein.

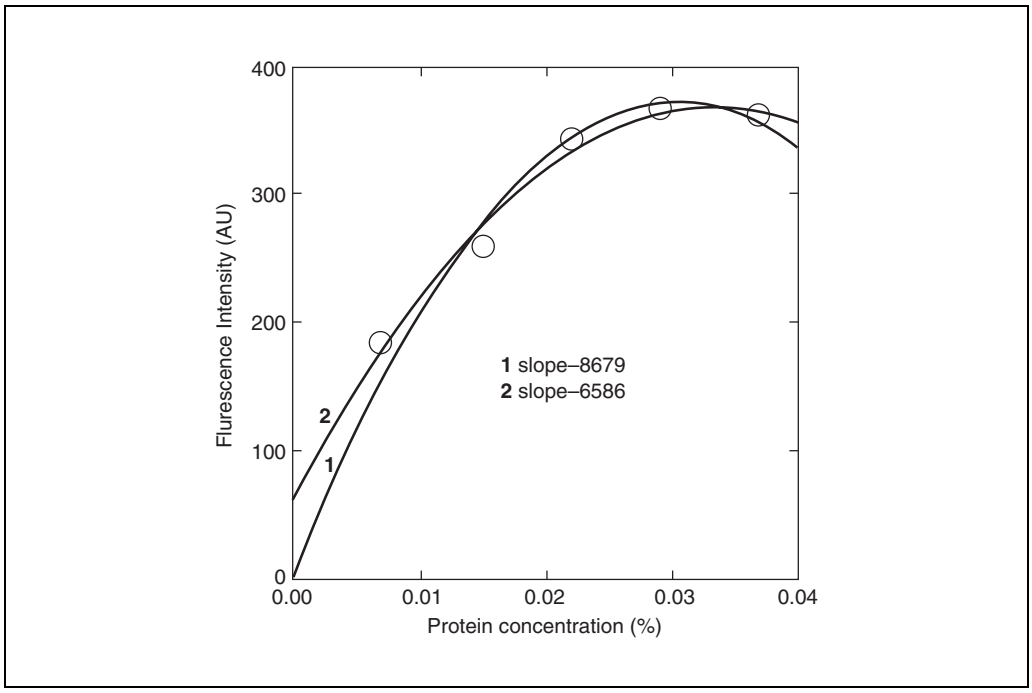

Figure B5.2.1 CPA hydrophobicity of bovine serum albumin. A quadratic model was fitted to the plot of net RFI versus protein concentration. The data point for the blank (buffer + probe) was included in the regression analysis for curve 1, but not for curve 2. Curve 2 gives a larger *y*-intercept and less steep slope. From Nakai et al. (1996) with permission from Kluwer Academic Publishers.

PROBE SPECTROFLUOROMETRY USING DPH

ALTERNATE PROTOCOL 1

DPH has been used to eliminate the charge effects observed when using ANS (Tsutsui et al., 1986). Because the dissociation constant of ANS is on the order of 10^{-3} to 10^{-4} (equivalent to relatively strong organic acids such as butyric and caproic acids), it is highly soluble in water and pH changes have only a slight effect on the quantum yield. CPA is even better in this regard. Nevertheless, some charge effects cannot be ignored due to the presence of a very weak carboxylic charge on the measured S_0. DPH is a membrane probe that has been used as a popular hydrophobic probe for membrane proteins with phospholipids (*www.probes.com/handbook/*); however, its very low solubility in water restricts its utilization as a fluorescent probe for proteins. Reduction of charges in probes immediately translates to a loss in water solubility, which is one of the most difficult problems in measuring the absolute hydrophobicity of protein molecules based on purely hydrophobic-hydrophobic interactions. Hydrophobic interaction is an entropy-driven solvation effect, without being contaminated with charge effects. The following is one solution for this problem, although the analytical procedure is more complicated.

Additional Materials *(also see Basic Protocol 1)*

Corn oil/heptane solution (19 mg oil/ml heptane)
Nitrogen source
Protein solution
0.02 M phosphate buffer, pH 7.4 (*APPENDIX 2A*)
1,6-Diphenyl-1,3,5-hexatriene (DPH; Sigma)

22 × 93–mm flat-bottom test tubes
Ultra Turrax homoblender (Janke and Kunkel, VWR) or an equivalent ultrasonic homogenizer

1. Dissolve 10 µg DPH in 1 ml of corn oil/heptane solution in a 22 × 93–mm flat-bottom test tube and remove the solvent by flush evaporation with a stream of nitrogen onto the surface of the solvent layer.

   Alternatively, dissolve DPH (at this same concentration) in tetrahydrofuran and add directly to the aqueous protein solution. Similar hydrophobicity results can be obtained this way (author's unpub. observ.).

2. Add 10 ml of serial protein concentrations of 0.02% to 0.12% in 0.02 M phosphate buffer, pH 7.4. Include a control without DPH.

   All conditions should be the same for the samples and blanks.

3. Blend 1 min in an Ultra Turrax homoblender while cooling in ice water.

4. Centrifuge emulsion 30 min at 27,000 × *g*, room temperature.

   If the protein precipitates during centrifugation, decant the upper (organic) layer, blend the suspension in the bottom layer again, and then follow the rest of the protocol.

5. Dilute the bottom layer 20-fold with 0.02 M phosphate buffer, pH 7.4.

6. Standardize the spectrofluorometer (see Basic Protocol 1, step 4) using DPH in heptane.

7. Measure the RFI at each protein concentration at excitation and emission wavelengths (λ_{ex}, λ_{em}) of 366 and 450 nm, respectively.

8. Analyze data to determine S_0 (see Basic Protocol 1, step 7).

ALTERNATE PROTOCOL 2

PROBE SPECTROFLUOROMETRY USING PRODAN

More recently, prodan has also been used to eliminate the charge effects observed with ANS (Haskard and Li-Chan, 1998). Prodan has both electron-donor and electron-acceptor substituents within the molecule, and is thus much easier than DPH to dissolve in more polar solvents (e.g., methanol). As a result, a simpler procedure than that of DPH was feasible.

Additional Materials *(also see Basic Protocol 1)*

3 μM 6-propionyl-2-dimethylaminonaphthalene (prodan; Molecular Probes) in 0.01 M phosphate buffer, pH 7.0 (add a minimum amount of absolute methanol until completely dissolved; see *APPENDIX 2A* for buffer)
0.2% (w/v) protein solution in 0.01 M phosphate buffer, pH 7.0
Test tubes (1-cm i.d. × 10-cm long)

1. Place 8 ml of 3 μM prodan in each of six tubes.
2. Add 0, 10, 20, 30, 40, and 50 μl of 0.2% protein solution to each tube. Include a blank tube without prodan.
3. Standardize the spectrofluorometer (see Basic Protocol 1, step 4) using prodan in methanol.

   All conditions should be the same for samples and the blank.

4. Measure the RFI of each protein concentration with excitation and emission wavelengths (λ_{ex}, λ_{em}) of 365 and 465 nm, respectively.
5. Compute the net RFI for each of the five protein concentrations by subtracting the blank (tube without prodan).
6. Analyze data to determine S_0 (see Basic Protocol 1, step 7).

BASIC PROTOCOL 2

MEASURING DETERGENT BINDING CAPACITY

The sodium dodecyl sulfate (SDS) binding capacity reported by Kato et al. (1984) is described here. SDS is a popular, powerful agent for dissociating globular proteins. SDS weakens hydrophobic interactions within the protein molecules by flooding them with the hydrophobic hydrocarbon chains of SDS, thereby forming new hydrophobic interactions. This method is more popular than the Tween binding capacity method described by Lieske and Konrad (1994), probably because of the well-characterized reactions of SDS with proteins in the literature. The concentration used here, however, is so low that SDS binding may be restricted to protein surfaces by not only hydrophobic but also ionic interactions.

Materials

0.01 M SDS in 0.02 M phosphate buffer, pH 6.0
0.01% (w/v) protein solution in 0.02 M phosphate buffer, pH 6.0
0.02 M phosphate buffer, pH 6.0, (*APPENDIX 2A*)
$CHCl_3$
0.0024% (w/v) methylene blue solution (Sigma)

Dialysis tubing (MWCO 3500)
10-ml volumetric flask
Test tubes
Spectrophotometer and cuvettes

1. Add 7 µl of 0.01 M SDS solution to 10 ml of 0.01% protein solution (final 0.007 mM). Allow the solution to stand 30 min at room temperature.
2. Dialyze the solution in dialysis tubing at 3° to 4°C against 25 vol of 0.02 M phosphate buffer, pH 6.0, for 24 hr while stirring on a magnetic stirrer to remove unbound SDS.
3. To adjust a possible volume change during dialysis, air-blow the tubing from the outside to slightly evaporate the content at room temperature and make up to the volume using a 10-ml volumetric flask.
4. Transfer 0.5 ml dialysate to a test tube, add 10 ml $CHCl_3$, and mix.
5. Add 2.5 ml of 0.0024% methylene blue solution, mix, and then centrifuge 5 min at 50,000 × *g*, room temperature.
6. Measure absorbance of the lower layer of the mixture in a regular glass cuvette at 655 nm.

   The SDS detection method (steps 3 to 5) is from Epton (1948).
7. Determine the amount of SDS by repeating step 1 with 2, 4, 6, and 8 µl of the SDS solution and 10 ml of 0.02 M phosphate buffer, pH 6.0, then follow steps 4 to 6.

   The standard curve is drawn using a mol. wt. of 288.4 for SDS.
8. Express SDS binding capacity as µg SDS bound/500 µg protein (a relative value for comparison).

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

BASIC PROTOCOL 3

Reversed-phase (RP) chromatography is useful in assessing polarity differences of water-soluble proteins. As the stationary phase of an RP column is nonpolar, the distribution of proteins in this phase is controlled by affinity due to hydrophobic interaction with the protein molecules. The stronger the hydrophobicity, the larger is the elution volume. Sample protein in aqueous solvent at a high ionic strength is applied to the column to minimize ionic effects for binding with column material, then eluted by a gradient of decreasing salt concentration. Two methods with aromatic (Goheen and Engelhorn, 1984) and aliphatic (Szepesy and Rippel, 1992) columns are used. Selection of the two columns depends on whether aromatic or aliphatic amino acid residues in the protein sample are of interest as the main force for affinity to the column material.

Materials

Protein solution
Solvent A:
- 1.7 M ammonium sulfate in 0.1 M sodium phosphate buffer (*APPENDIX 2A*), pH 7.0 (for aromatic column)
- 2 M salt in 0.02 M Tris·Cl buffer (*APPENDIX 2A*), pH 7.0, adjusted with 0.1 M HCl (for aliphatic column)

Solvent B:
- 0.1 M sodium phosphate buffer, pH 7.0 (for aromatic column)
- 0.02 M Tris·Cl buffer, pH 7.0, adjusted with 0.1 M HCl (for aliphatic column)

HPLC system with analytical column and an appropriate guard column:
- 75-mm i.d. × 75-mm long TSK-Phenyl 5-PW analytical column (Beckman; aromatic)
- 4.1-mm i.d. × 250-mm long Syncropak-Propyl (Synchrom; aliphatic) *or* 4.6-mm i.d. × 100-mm long Spherogel CAA-HIC polyether (Beckman; aliphatic)

1. Dissolve 1.0 mg protein in 1.0 ml water.
2. Apply protein sample to the analytical column.
3. Elute using a linear gradient from 100% solvent A to 100% solvent B at a flow rate of 1.0 ml/min.
4. Calculate the apparent capacity factor as $k_g = (t_g - t_0)/t_0$, where t_g is the retention time and t_0 is the mobile phase hold-up time.

BASIC PROTOCOL 4

DETERMINATION OF CONTACT ANGLE

Contact angle is the angle of saline drop at the contact edge built on the surface of a hydrated protein film formed on a glass slide; the larger the angle, the greater the protein hydrophobicity. This method provides a physical approach with minimal chemical treatments. Once the facility is set up, it is simple to carry out. According to van Oss et al. (1981), the surface tension of protein solutions is contributed by Lifshitz-van der Waals (LW) forces and Lewis acid-base (AB) forces. An example of application to food proteins can be seen in Nasir and McGuire (1998).

Materials

1% or 2% (w/v) aqueous protein solution in pH 7.3 buffer containing 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 1.0 g EDTA in 1 liter
45-mm anisotropic cellulose acetate membrane
Small telescope with cross hairs attached to a goniometer (an angle-measuring device) or enlarged photograph

1. Ultrafilter 30 ml of 1% or 2% protein solution through a 45-mm anisotropic cellulose acetate membrane under vacuum without stirring until the flow rate slows down considerably (almost to a stop).
2. Begin to air-dry the membrane with the built-up hydrated protein layer.
3. At various time intervals during the drying process, apply 10-µl drops of isotonic saline to different locations on the surface of the protein layer and measure the contact angle using either a small telescope with cross-hairs attached to a goniometer, or an enlarged photograph taken from a vertical side of the slide glass.

   Initially, when the protein layer is still wet, contact angles close to 0° are obtained. As drying proceeds, there will be a quick increase in contact angle, and then a plateau angle (θ) will be reached (typically within 60 min).

4. Calculate the surface tension (γ_{SV}) of the protein (solid/air), which is a measure of hydrophobicity (Keshavarz and Nakai, 1979) based on Young's relationship:

$$\gamma_{SV} - \gamma_{SL} = \gamma_{LV} \cos\theta$$

where γ_{LV} is a known value for the surface tension of liquid/vapor, and γ_{SL} is a known value for the surface tension of solid/liquid.

The advanced contact angles of droplets of three different liquids—i.e., water, glycerol, and α-bromonaphthalene (with reducing polarity in this order)—on the protein surface are measured. Based on Young's equation and the known surface tensions of the three liquids, the contact angles are then used to calculate the contribution of LW and AB interactions to the surface tensions of the liquids on the layered protein. Contact angle instruments including a computer for data processing are commercially available (Kruss, http://www.kruss.de).

DETERMINATION OF HYDROPHOBIC PARTITION

BASIC PROTOCOL 5

Among many methods proposed for hydrophobic partition, only the method of Shanbhag and Axelsson (1975) is described here. The principle of this method is phase separation by partitioning the hydrophobic upper phase (palmitate is used as it is the most abundant saturated fatty acid in food fat) and the hydrophilic lower phase of proteins. A protein will be separated more into the top layer when it is hydrophobic. The method used by Keshavarz and Nakai (1979), based on Shanbhag and Axelsson (1975), is described in this protocol.

Materials

Protein of interest
100 mM K_2SO_4/2 mM phosphate buffer, pH 7.1 (see *APPENDIX 2A* for buffer)
40% polyethylene glycol (PEG) as Carbowax 6000 (Union Carbide)
PEG-palmitate (see Support Protocol)
20% dextran T70 (mol. wt. 70,000) in 100 mM K_2SO_4/2 mM phosphate buffer, pH 7.1
0.02% SDS in 0.1 N NaOH

10-ml centrifuge tubes
UV spectrophotometer

Prepare two-phase systems

1. Place 5 to 15 mg protein in each of two 10-ml centrifuge tubes and dissolve in 0.2 ml of 100 mM K_2SO_4/2 mM phosphate buffer, pH 7.1, by shaking in a vortex mixer. Make up two 10-ml centrifuge tubes of blank without protein.
2. Add 1.6 g of 40% PEG to one tube (system I) and a similar amount of PEG-palmitate to the other (system II).
3. Add 3.2 g of 20% dextran stock to each and mix for 10 min at 40°C.
4. Mix by gentle inversion and then centrifuge 5 min at 1500 × *g,* room temperature.

Perform assay

5. Withdraw a 2-g aliquot from each sample phase and dilute 5- to 10-fold with water (0.02% SDS in 0.1 N NaOH for PEG-palmitate phase). Measure the absorbance at 280 nm against the appropriate blank.
6. Calculate the partition coefficient, $\Delta\log K$:

$$\Delta\log K = \log[C_u/C_l]_I - \log[C_u/C_l]_{II}$$

where C_u and C_l are the concentration of the partitioned protein in the upper and lower phases, respectively, for the systems with (I) and without (II) the ligand derivative of PEG.

SUPPORT PROTOCOL

SYNTHESIS OF PEG-PALMITATE

This procedure was originally described by Shanbhag and Johansson (1974) and yields an ~60% substitution of the total hydroxyl groups in PEG.

Materials

Polyethylene glycol (PEG) as Carbowax 6000 (Union Carbide)
Toluene
Triethylamine (TEA)
Palmitoyl chloride
Whatman no. 1 and 4 filter paper

1. Dissolve 100 g PEG in 600 ml water.
2. Add 100 ml toluene, evaporate off the solvent, and repeat. Add 2 g TEA.
3. Dissolve 2.5 g palmitoyl chloride in 25 ml toluene and then add dropwise to TEA/PEG solution (from step 2).
4. Reflux 15 min at room temperature and filter through a Whatman no. 4 filter paper under gravity.
5. Precipitate by cooling to 3°C and collect the crystals by vacuum filtration through a Whatman no. 1 filter paper.
6. Recrystallize product from absolute ethanol two times by first dissolving as much PEG-palmitate as possible in warm (40° to 50°C) ethanol and then cooling it off at 4°C to crystallize.

COMMENTARY

Background Information

Protein hydrophobicity defined

There are two definitions of protein hydrophobicity: average hydrophobicity and surface hydrophobicity. The average hydrophobicity was defined by Bigelow (1967) as the total hydrophobicity of all amino acid residues comprising a protein divided by the number of amino acids in the protein. There is no standard definition of surface (or effective) hydrophobicity except the concept that there must be hydrophobic regions on the molecular surface that play an effective role in protein function. Readers who are interested in a more detailed discussion are referred to Nakai and Li-Chan (1988).

Many scales, either empirical or measured, have been proposed for the hydrophobicity of amino acid residues in proteins (Nakai and Li-Chan, 1988). The most extensive study on the hydrophobicity index of amino acids was published by Wilce et al. (1995). The authors derived four new scales of coefficients from the reversed-phase high-performance liquid chromatographic retention data of 1738 peptides and compared them with 12 previously published scales.

It has been accepted that nonpolar or hydrophobic groups should be restricted to the interior of a folded molecule to achieve the minimum free energy in the folding of macromolecules, thus not being exposed to the solvent water. In reality, however, crystallographic studies of the three-dimensional structures of proteins have revealed that many hydrophobic groups are at least partially exposed on the surface of proteins. The resulting surface-exposed hydrophobic sites play a key role in intermolecular interactions such as binding with small ligands, protein-lipid interactions, and interactions with other biological macromolecules.

Role of hydrophobicity in physicochemical properties

Klein et al. (1986) reported the compositional or physicochemical properties (attributes) of amino acid sequences in 1603 protein sequences to establish a classification system for 26 protein functions. These results showed that three or four attributes were generally sufficient to distinguish each of the 26 functional categories from the remainder of the database. The attributes used were related to hydrophobicity, charge and its distribution (frequency or

occurrence), a periodicity of appearance, and secondary structure parameters.

Kato and Nakai (1980) found that the effective hydrophobicity measured by using *cis*-parinaric acid (CPA; see Basic Protocol 1) could explain surface properties of proteins that were not necessarily directly correlated with the average hydrophobicity. Since then, the importance of surface hydrophobicity in protein functions has been widely recognized. As a recent example, the effect of oxygen radical–dependent oxidation resulted in an increase in protein surface hydrophobicity that was highly correlated with hydrophobicity changes by aging (Chao et al., 1997). Although an increase in methionine sulfoxide and dityrosine during aging resulted in a conformational change due to cross-linking, the mechanism of the increase in surface hydrophobicity is still controversial. For food proteins, the author has reviewed the measurement of surface hydrophobicity (Nakai et al., 1996). Unfolding of protein molecules, thereby exposing interior hydrophobic sites prior to cross-linking formation, such as molten globule formation and gelation due to a variety of reasons have been reported.

Comparison of different methods

ANS (see Basic Protocol 1) has been the most popular hydrophobic probe for the determination of surface hydrophobicity of proteins. Its dimeric form bis-ANS, which has a greater quantum yield in nonpolar environments by binding more strongly with proteins than the ANS monomer, is occasionally used for the same purpose (Das and Surewicz, 1995). These effects permit the observation of depolarization by energy transfer among the bound fluorophores, which can be used to estimate the distribution of the ligands among the protein molecules (Farris et al., 1978).

Cardamone and Puri (1992) stated that ANS binding and resultant K_a measured by a Scatchard plot or Kloz plot (and to a lesser extent quantum yield) may be used as a measure of the relative surface hydrophobicity of proteins. Titration of protein solutions with increasing concentrations of the fluorescent probe can provide information on both the number and the affinity of binding sites. This may be useful in determining whether the high value of fluorescence resulted from the presence of many binding sites of only moderate hydrophilic character, or from the existence of a high-affinity site with considerable hydrophilic character.

The application of fluorescence probes to the investigation of the proximity of the probe binding site(s) in aromatic groups has also been assessed by measuring the efficiency of energy transfer. Transfer of excitation energy from aromatic chromophores to a bound probe at an adjacent hydrophobic site causes quenching of the intrinsic fluorescence of the aromatic side chain. This transfer efficiency can also be used as an index of surface hydrophobicity (Gatti et al., 1998) but it is yet to be widely recognized.

Another group of anionic fluorescent probes is the fatty acid analogs, including cis-parinaric acid (CPA) and trans-paranaric acid, which have been widely used as probes for proteins and biological membranes (Sklar et al., 1977). Therefore, this original CPA method was immediately extended to food proteins by Kato and Nakai (1980). The parinaric acids are nonaromatic fluorophores; their similarity to native fatty acids, their inability to fluoresce in water, and their good Stokes' shift characteristics are advantageous to probe for hydrophobic regions (patches) that may be important in protein-lipid interactions in food systems. Good correlations were obtained between the relative hydrophobicity values of proteins determined by CPA fluorescence and properties related to protein-lipid interactions such as interfacial tension and emulsifying activity (Kato and Nakai, 1980). This hydrophobicity is an aliphatic hydrophobicity that can be differentiated from the aromatic hydrophobicity using aromatic fluorophores such as ANS (Hayakawa and Nakai, 1985). The latter may be useful in investigating protein-aromatic interaction, such as those with retinol and aromatic flavor compounds. ANS and CPA were both used for elucidation of heat-induced gelation of myosin (Boyer et al., 1996).

Limitations in using anionic probes such as ANS and CPA to determine protein hydrophobicity include the possibility that electrostatic as well as hydrophobic interactions may contribute to the interaction between the protein and the probe (Greene, 1984). The use of charged but neutral probes (having both electron donor and acceptor groups; e.g., prodan) or uncharged probes (e.g., DPH; Davenport, 1997) may circumvent this problem. However, the nonpolar nature of DPH restricts its solubility in aqueous systems and thus limits its use as a probe for protein solutions. About the same RFI slopes for DPH were obtained as those for ANS for 11 proteins, except for heated bovine serum albumin, which had a much lower value (Tsutsui et al., 1986). The low solubility of

weak acids, like CPA, also limits use at pH <5, and the interpretation of results should be made with caution due to an increased quantum yield of CPA in its undissociated form at acidic pHs.

Boatright and Hettiarachchy (1995) reported that solubility improvement in soy protein resulted from preventing its oxidation by adding antioxidants and corresponded with the increase in total protein surface hydrophobicity as determined by the SDS binding method (see Basic Protocol 2). This hydrophobicity was not the total protein hydrophobicity but is similar to the exposable hydrophobicity, S_e, which is S_0 measured in the presence of SDS (Townsend and Nakai, 1983). This change in the exposure of hydrophobic sites in protein molecules is dependent on the concentration of SDS used in the analysis. One of the advantages of the SDS binding method is that there is no need for an expensive spectrofluorometer.

Based on the principle of partition chromatography, the retention volume has been considered a good index of the polarity of solutes. According to the solvophobic effect theory of Melander and Horváth (1977), the retention time of peptides depends mainly on their behavior on the surface of nonpolar columns, thus being reasonable to use hydrophobicity coefficients of amino acid residues in peptides. However, in the case of proteins, most solvents being used in reversed-phase chromatography may denature proteins. Therefore, hydrophobic interaction chromatography (see Basic Protocol 3) using negative ionic gradients in aqueous eluents is preferred for determination of protein hydrophobicity. Despite the milder elution conditions being used, denaturation of protein at high salt concentrations (Barford et al., 1988) and on the hydrophobic surface of the stationary phase (Szepesy and Rippel, 1992) cannot be totally ignored. Standard chromatographic protocols to assess surface hydrophobicity of native states of protein molecules are still unavailable to protein chemists.

Using contact angle (see Basic Protocol 4), the quantitative determination on the basis of the Lifshiz-van der Waals and electron donor-acceptor of Lewis acid-base interactions contributing to surface tension was extended to proteins (van Oss et al., 1981). Despite its relative simplicity, the method has not gained popularity in the application to food proteins directly, probably because of the lack of similarity with proteins in solutions as this method uses a semi-dried form of proteins. However, investigation of the quality of packaging material, such as permeation and absorption, may be a good example of the application of this method.

The most popular method for measuring the polarity of a solute entails determination of the distribution constant between water and a water-immiscible solvent, e.g., octanol. However, because there is difficulty in dissolving proteins in the solvent, a two-phase aqueous system was developed (Shanbhag and Axelson, 1975). Albertson (1986) reported the construction of various aqueous phase systems for partitioning proteins, other macromolecules, and even cells. Recently, simpler aqueous biphase systems were selected for hydrophobic partitioning of proteins (Hachem et al., 1996). However, because of restrictions similar to those for HIC, as discussed above, it may be premature to replace the method used in Basic Protocol 5. The definition of hydrophobicity is based on the polarity of chemical compounds, which is closely related to the distribution between solvents of different polarities. This theory is similar to the elution mechanism of phase distribution chromatography as well as phase partition. However, complexity in the partition system and procedure hampers the broad use of the phase partition approaches.

Comparison of protein hydrophobicity measured using different methods is tabulated in Nakai et al. (1996).

Other available methods

A number of spectroscopic methods provides useful information on protein structure and the environment of constituent amino acid residues. The intrinsic fluorescence spectrum of proteins (*UNIT B3.6*) is primarily attributed to the aromatic amino acid residues. In practice, tryptophan fluorescence is the commonly studied aspect of the spectrum, because phenylalanine has a low quantum yield and tyrosine fluorescence is frequently weakened due to quenching by ionization or by interactions with amino, carboxyl, or tryptophan residues. Three classes of tryptophan residues have been reported (Burstein et al., 1973), namely buried, exposed, and have limited contact with water, therefore probably being immobilized at the surface. The wavelengths of maximum emission for each class of tryptophan residue are 330 to 332 nm, 350 to 353 nm, and 340 to 342 nm, respectively. Recently, intrinsic fluorescence of hemoglobins and myoglobins were thoroughly reviewed (Gryczynski et al., 1997).

Fourth-derivative spectrophotometry in the UV region can be used to assess the contents of aromatic amino acid residues and thus enable

measurement of changes in polarity of the microenvironment around the chromophores (Mozo-Villaria et al., 1991).

The possibility of using NMR cross-saturation (Arteaga, 1994) and Raman spectroscopy to measure C-H stretching regions (Howell et al., 1999) were discussed for assessing hydrophobic interactions of proteins. However, protein hydrophobicty either total or surface cannot be solidly defined; it is readily affected by only slight circumstantial changes, such as pH, temperature, and concentration, which are extremely difficult to control precisely.

Further studies needed

Unlike small molecular compounds, the compactness of protein molecules must affect the accessibility of reactive groups to solvents, namely hydrophobic and charged groups. Upon structure changes due to alterations in microenvironments is, therefore, important for elucidating protein functions. Because of the close relationship among the important parameters that are involved in the mechanisms of protein functions, i.e., hydrophobic, electronic, and structure-related parameters, a high correlation coefficient in a linear regression analysis may not always be a better criteria to demonstrate the importance of these parameters. For both aliphatic and aromatic hydrophobicities, the selection of a single cause for elucidating function-related phenomena is almost impossible. Continual improvement in the analytical methodology is required to exclusively define protein hydrophobicity for a precise explanation of the structure-function relationships of food proteins. The complex nature of molecular structures may increasingly require more reliable nonlinear algorithms such as partial least squares (PLS) regression or artificial neural networks (ANN) to analyze structure-function relationships of food proteins (Arteaga and Nakai, 1993; Burden and Winkler, 1999; Cronin and Schultz, 2001).

Another recent trend is to show the importance of hydrophobic profiles rather than molecular hydrophobicity. Giuliani et al. (2002) suggested nonlinear signal analysis methods in the elucidation of protein sequence-structure relationships. The major algorithm used for analyzing hydrophobicity sequences or profiles was recurrence quantification analysis (RQA), in which a recurrence plot depicted a single trajectory as a two-dimensional representation of experimental time-series data. Examples of the global properties used in this RQA were thermal stability, protein-peptide interactions, and folding behavior.

Critical Parameters and Troubleshooting

Chemical structure

Although similarity in chemical structures between compounds does not always imply greater solvation effects, at least it is likely that compounds in similar structures have similar polarity ranges. Based on this rule, the existence of two hydrophobicities (namely aliphatic and aromatic) and despite the fact that it is extremely difficult to distinguish them, the solvation effects cannot be measured by discriminitating between the two groups. A good example is the difference in surface hydrophobicity between bovine serum albumin (BSA) and ovalbumin reported by Haskard and Li-Chan (1998). Greater differences in S_0 values of the two proteins were produced by using probes less charged than ANS. However, it is unknown whether a similar difference measured by CPA may be due to a high charge effect of this compound, as the appreciably lower dissociation constant of CPA than ANS is apparent. This may implicate that ANS charges contribute less to the true surface hydrophobicity than CPA charges.

Structure-function relationships

From a practical point of view, any surface hydrophobicity values measured that show highly significant relationships with specific functions, should be useful in elucidating the mechanisms of functions. However, since in elucidating most of the functionalities, hydrophobic and charge effects are difficult to separate; in reality, they are countercorrelated. As a result, it is possible that some surface hydrophobicities measured by methods including both effects may show better correlations with the funcitonsality, mostly by chance. Therefore, these parameters, e.g., correlation coefficient of functionality versus surface hydrophobicity of a probe, cannot be a good criteria to determine the correctness of representing the true surface hydrophobicity of proteins. There is still an unanswered question, which of the surface hydrophobicities measured as S_0 computed from the initial slope or the saturated fluorescence value appeared as the plateau of titration curves (Iametti and Bonomi, 1993) correlate better with functional properties. This question should be answered in the future.

Spectrofluorometry

Contaminants in protein samples, including lipids and fluorescent material, interfere with the fluorescence intensity measurements. For some probes, especially weakly acidic dissociable probes such as *cis*-parinarate, extreme pH cannot be used, as the quantum yield is different from that in an undissociated form at low pH. Also, sometimes the purity of probes critically affects the fluorescence intensity, thus requiring purification of the probes.

Buffers

Phosphate buffers are most frequently used to dissolve food proteins because they mostly exist in food systems at neutral pH and because of the strong buffering capacity of phosphate buffers at broad pH ranges.

Anticipated Results

Protein hydrophobicity has been most frequently expressed as relative values measured by the methods used, since no standardized unit has ever been established. These relative values are incorporated directly into correlation studies with protein functions. Therefore, the correlation coefficient of a measured functionality against the counterpart predicted from the measured hydrophobicity is the most reliable parameter to use for comparing different methods for hydrophobicity measurement.

Time Considerations

Most fluorescence probe methods can accommodate more than ten samples per day. However, other methods such as detergent binding, hydrophobic interaction chromatography, and hydrophobic partitioning can accommodate only one or two protein samples a day.

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Key References

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A compilation of analytical methods for hydrophobic interactions in food systems. Although this book is outdated, there has been no recent publication of a similar book. The alternative is recent review articles of each analytical method.

Internet Resources

http://www.probes.com

Molecular Probes Web site, which provides key information and additional references.

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Water Retention Properties of Solid Foods

"Water retention properties" is a term that is meant to represent a set of methods that have previously been collectively referred to as water holding capacity (WHC). The various water holding capacity tests used in the literature may use very different methods that lead to very different results. There are many available methods, with no standard or universally accepted methods for measuring water retention in food materials. This unit presents protocols for (1) expressible moisture by centrifugation using filter paper (see Basic Protocol 1), (2) drip loss by simple collection of moisture (see Basic Protocol 2; the same test can be used for cook loss, thaw loss, and so on), and (3) water uptake ability using the Omnimixer and centrifugation (see Basic Protocol 3). These protocols are presented as examples of rapid methods for measuring the water retention of various solid food materials, particularly flesh food material, for which they were developed. See Background Information for a discussion of these various methods. Also see *UNIT B5.1* for a discussion of important considerations when choosing a protein functionality test.

The expressible moisture test is a quick and simple method to measure the amount of water that might be expelled from the sample under the conditions of the test. By using a centrifuge, the actual water loss of the material is calculated rather than a relative "ring diameter" as in the classical Carver press method (Forbes et al., 1974; Lee and Patel, 1984). The water uptake ability assay is a very quick, easy, and relatively inexpensive test to determine some important properties of a solid food. It is important to choose the appropriate "solution" conditions for the added liquid. Many of the experimental parameters are somewhat arbitrary, although chosen only after preliminary experiments designed to optimize the test. This method is designed so that expressible moisture and water uptake ability can be compared using the same arbitrary experimental conditions.

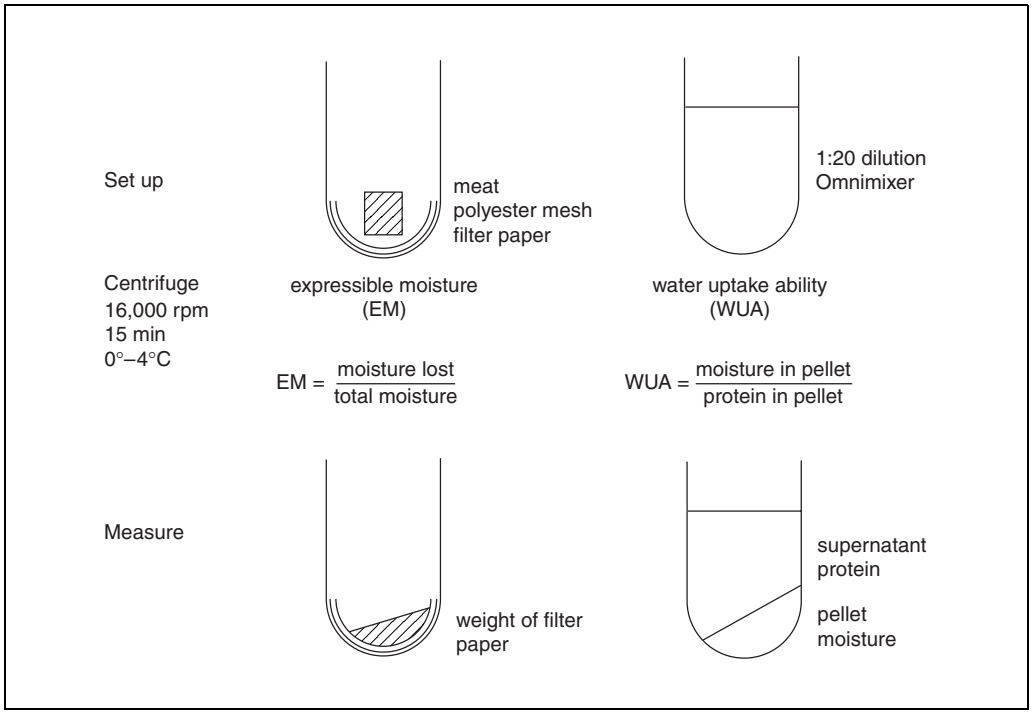

Figure B5.3.1 A comparison of water retention properties: expressible moisture versus water uptake ability. Reprinted from Regenstein (1984) with permission from the American Meat Science Association.

Contributed by Joe M. Regenstein

Current Protocols in Food Analytical Chemistry (2003) B5.3.1-B5.3.9

Although the conditions used for the measurement of both expressible moisture and water uptake ability are arbitrary, the two can be done under similar conditions. One of the real benefits of the two tests, with arbitrarily defined working parameters, is that they can in theory be done together, e.g., four samples for expressible moisture and four for water uptake ability can be placed in the same eight-cell centrifuge. Thus, the differences observed in these two tests reflect real differences in the underlying properties of the materials being measured. This is illustrated in Figure B5.3.1.

Drip losses, cooking/processing loss, and thaw loss are related to, but different from, expressible moisture. In these measurements, the moisture loss that is measured is the amount lost without the application of any force. Thus, the liquid that is expelled can be collected by carefully "pouring off" the excess liquid. Placing the food sample in a funnel and collecting the drip in a graduated cylinder is often the easiest way to practically accomplish this task.

BASIC PROTOCOL 1

MEASUREMENT OF EXPRESSIBLE MOISTURE

Expressible moisture measures the water expressed from a material under a force. Its measurement requires centrifugation of samples on filter paper to remove the released moisture. The filter paper needs to be weighed before and after centrifugation. Weight loss of the sample can also be measured by weighing before and after centrifugation.

Materials

Food sample (~1.5 g)
Whatman no. 50 (7-cm diameter) and no. 3 (5.5-cm diameter) filter papers
Scale
Polyester mesh, 690 mesh per cm^2 (Henry Simon)
50-ml polycarbonate centrifuge tubes
Centrifuge with eight-cell rotor to hold 50-ml polycarbonate centrifuge tubes (e.g., Sorvall SS-34 rotor and Nalgene tubes)

1. Weigh one piece of Whatman no. 50 filter paper and three pieces of Whatman no. 3 filter paper.
2. Weigh food sample.

   Larger samples may be needed for drier material. In some cases, grinding prior to measuring the sample may also be appropriate.

3. Cut one circle of polyester mesh so that it is slightly smaller than the Whatman no. 50 filter paper.
4. Place weighed food sample on mesh screen.
5. Place food sample plus mesh screen on the four pieces of preweighed filter paper (Whatman no. 50 then three pieces of no. 3) and fold into essentially a thimble shape with the food sample in the center.
6. Place the entire package in a 50-ml polycarbonate centrifuge tube.
7. Centrifuge 15 min at 31,000 × *g* (16,000 rpm in a Sorvall SS-34 rotor), 4°C.
8. Remove sample from the centrifuge tube and carefully remove the food sample and polyester mesh.
9. Measure the weight gain of the four pieces of filter paper.
10. Calculate the expressible moisture (EM) as the weight of the filter paper after centrifugation (W_{F2}) minus the weight of the filter paper before centrifugation (W_{F1}) divided by the weight of the initial sample (W_S). To express as a percentage, multiply by 100.

11. Determine the total moisture content of the initial sample (*UNIT A1.1*) in order to calculate the moisture retained, which is the total initial moisture minus the expressible moisture.

DRIP LOSS OF COOKED, FROZEN, OR OTHERWISE PROCESSED FOODS

BASIC PROTOCOL 2

Drip loss is used to measure the natural loss of moisture by a food material. The sample needs to be weighed before and after the drip is removed. The time allowed for drip must be standardized. Alternatively, a graduated cylinder can be used to collect the liquid. Generally, a funnel is used to hold the sample.

Materials

Food sample
Funnel of sufficient size to hold entire sample
Graduated cylinders appropriate to sample size
Plastic cling film

1. Carefully weigh out an appropriate sample (e.g., 5 to 50 g) and place into the top of a funnel.

   This test will tolerate a fairly wide range of sample sizes.

   Often one will try to make sure that the sample is not the shape of the funnel, i.e., a cylinder-shaped sample is not desired, as some air space between the funnel and the sample is needed so that the liquid does not travel through the sample. Ideally, the top of the sample should be shaped so that any liquid will run off. Often a wire or screen is used to hold the sample so that moisture can drain.

2. Place the funnel into a graduated cylinder.

3. Cover the funnel and sample with plastic cling film and place entire setup in a 0° to 4°C cold room.

   It is important to cover the entire assembly to prevent evaporative losses.

4. Leave for 24 hr in the cold room.

   As a time-dependent phenomenon is measured, constant time is important. However, for some purposes, adjusting the waiting time might be appropriate. For cook loss in particular, a much shorter time may well give a constant result. The experimental time should always be reported.

5. Measure the amount of drip.

   This is normally measured as a volume, and it is assumed that 1 ml liquid = 1 g. Obviously, for greater accuracy, the weight of the liquid could be determined. In some cases, one may also want to distinguish between aqueous weight/volume loss and lipid weight/volume. One could also measure the weight loss of the sample directly.

6. Calculate drip loss (%) = $V_D/W_S \times 100$, where V_D is drip volume and W_S is the initial weight of the sample.

7. To further characterize the drip solution, measure its protein/solids content, e.g., by performing a colorimetric assay for total protein (e.g., Lowry; *UNIT B1.1*) and possibly analyzing by electrophoresis (*UNIT B3.1*) or chromatography (*UNIT B4.2*) to determine which proteins are actually lost in the drip solution.

8. To determine the percentage of the total available moisture lost, first determine the moisture in the initial sample (*UNIT A1.1*). Then calculate the percentage of the total available moisture lost by dividing the percent drip loss by the total percent moisture content of the sample.

WATER UPTAKE ABILITY

Water uptake ability is a measure of the amount of water that a sample can imbibe under specified centrifugation conditions. The amount and composition of the sample need to be known. Samples need to be blended in the presence of excess liquid. After centrifugation, the amount of protein in the supernatant (i.e., protein lost from the sample) and the volume of supernatant can be measured to accurately determine water uptake ability. Alternatively, WUA can be measured from the pellet itself. Both calculation methods are presented here.

Materials

Sample
Distilled, deionized water or buffer solution that is appropriate for the experiment

50-ml centrifuge tubes
Omnimixer (Sorvall) with adapter for 50-ml centrifuge tubes
Centrifuge (with a Sorvall SS-34 rotor or equivalent)
Graduated cylinder
Drying oven for moisture determination

Additional reagents and equipment for Kjeldahl, Lowry, or other protein determination method (*UNIT B1.1*)

Perform uptake experiment

1. Carefully weigh out ~1.7 g sample and place in a 50-ml centrifuge tube.
2. Take a second sample of an appropriate size for protein determination.

   Often the amount of protein in the sample is the basis for comparative measurements (e.g., for meats). In this case, protein determination is needed. Either a Kjeldahl or other appropriate protein assay (UNIT B1.1) can be used.

3. Add ~18 volumes (relative to sample weight) of distilled, deionized water or buffer solution that is appropriate for the experiment.

   A preliminary study (Regenstein and Rank Stamm, 1979) showed that an ~20-fold liquid excess was needed to obtain results that were not dependent on the ratio of solids to liquid. The 2× extra solution can be saved to rinse off the Omnimixer blades.

   The actual solution chosen depends on the needs of the experiment. For meat samples, it is recommended that the solution contain a buffer so that samples can be measured at a consistent pH. In some practical situations, however, no buffer should be present since the impact of natural pH differences in the samples is part of the desired result.

4. Blend 30 sec in an Omnimixer at a speed setting of 5. Use the remaining buffer (i.e., to a total of 20 volumes) to rinse the Omnimixer blades into the sample.

   The use of an Omnimixer is specifically recommended for this method (see Background Information).

5. Centrifuge samples 15 min at 31,000 × *g* (16,000 rpm in a Sorvall SS-34 rotor), 4°C.
6. Carefully decant supernatant into a graduated cylinder.

   In most samples, a sharp separation is obtained. Occasionally, however, the distinction between precipitate and supernatant is not clear, and it is important to use consistent judgment in recovering the supernatant.

   WUA can be determined by measuring the fluid remaining in the supernatant and the amount of protein lost to the supernatant (steps 7a to 9a), or by measuring the dry and wet weight of the pellet itself (steps 7b and 8b). The latter is a much more accurate procedure but requires ~24 hr for drying.

Determine WUA

From water in supernatant:

7a. Measure the amount of liquid and its protein content. Multiply the volume (ml) of the recovered supernatant by the protein concentration (g/ml) to determine the amount of protein that is soluble.

> *This solubility reflects the conditions that were used. Most solubility measurements (e.g., Regenstein and Regenstein, 1984; Morr et al., 1985) are designed for powders and reflect additional experimental controls. This is a more "operational" measurement of solubility.*

8a. Subtract the total soluble protein from the total initial protein (step 2) to determine the amount of insoluble protein.

9a. Subtract the volume of the recovered supernatant from the total water added to determine the volume of water uptake. Express water uptake ability (WUA) as the volume of water uptake divided by the amount of insoluble protein.

From water in pellet:

7b. Take the entire pellet and weigh it. Dry the pellet under constant moisture (see *UNIT A1.1*) and weigh it again.

8b. To obtain the actual amount of moisture uptake (WUA) by a given amount of solids (dry weight), subtract the dry weight of the pellet from the wet weight of the pellet and divide by the dry weight.

COMMENTARY

Background Information

Terminology

"Water retention properties" represent a set of methods that have previously been collectively referred to as water holding capacity (WHC). However, the various water holding capacity tests used in the literature may use very different methods. These methods are different enough that they are not measuring the same properties. In addition to addressing the methodological issues, a new vocabulary has been developed to bring some clarification to the field. The various types of measurements or properties covered by the term "water retention properties" includes the following:

Drip. Water that naturally exudes out of a material.

Expressible moisture. Water that can be squeezed out of a material by application of an external force.

Water uptake ability. Excess additional water that a material can imbibe. (The author recognizes the contribution of the USDA Regional Hatch Project NE-123 for this term.)

Thaw drip, cook loss, and other terms. Water that is naturally released because of a processing step. Such terms also identify the process that would impact on moisture loss.

Expressible moisture

Expressible moisture (EM) measures the water that can be squeezed out of a material, generally under a force. Traditionally, the Carver press was used with meats (Forbes et al., 1974; Lee and Patel, 1984). A piece of meat was placed between large pieces of filter paper. The press squeezed out the moisture, and the investigator measured the diameter of the wet circle on the filter paper. One of the problems with the Carver press method is ensuring the use of consistent pressure each time. Also, one can only measure a single sample at a time. Because of the arbitrariness of the conditions, the exact relationship to a real sample is not known. The actual amount of moisture loss is also not determined.

As an alternative, centrifugation can be used. The major issue in this approach is how to collect the expressed moisture. One way, as presented in this unit, is to collect the moisture on filter paper and measure either the weight gain of the filter paper or the weight loss of the sample. The material can be put into a few pieces of filter paper and centrifuged. As a practical matter, the author has found that a piece of nylon mesh placed between the meat sample and the filter paper make it easier to remove the meat sample after centrifugation

(i.e., one does not need to tease the sample off of the filter paper).

Another way to measure the expressible moisture in a centrifuge is to use some type of filter system so that the free solution is collected below the sample. This involves specialized equipment in each centrifuge tube that subsequently needs to be cleaned.

Any test involving a centrifuge needs to be standardized for centrifuge speed, time, temperature, and sample size. In almost all cases, these decisions are somewhat arbitrary. Obviously, higher centrifuge speeds will remove more water.

In some cases, it is desirable to study the influence of various "additives" on the expressible moisture. To standardize this testing, solutions of test compounds were added to the food sample at an arbitrary 10% by weight and samples were mixed well but gently. The controls included both untreated samples and samples treated with 10% water. All solutions were added at 10% by weight. It is necessary to be clear when expressing the results whether the concentration of the added material is being expressed in terms of its concentration in the aqueous solution added or in terms of its final concentration in the sample (the latter is preferred).

Water uptake ability (WUA)

The other type of water retention measurement is water uptake ability. The basic question that this test tries to answer is, what is the amount of water a material can retain in the presence of an excess of solution? One might consider this measurement as the "sponge" capacity of a food material. This property depends mainly on the properties of the insoluble phase and on the physical state of the food, i.e., the degree of particle disintegration. It is thus a question that can only be asked of an insoluble or partially insoluble material.

After centrifugation, the insoluble material will increase in volume if it takes up water. Excess water will constitute the supernatant.

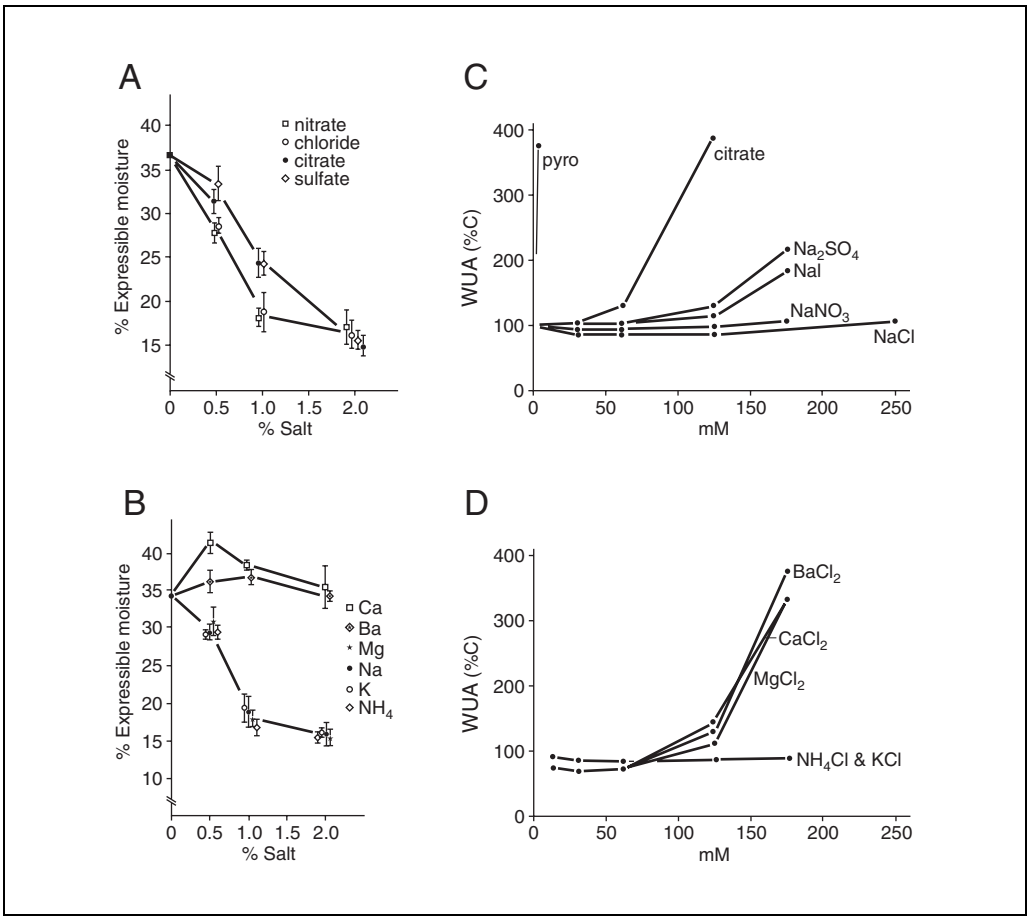

Figure B5.3.2 A comparison between expressible moisture and water uptake ability (WUA) measurements for different cations and anions in fish muscle. Reprinted from Regenstein (1984) with permission from the American Meat Science Association, and from Regenstein and Regenstein (1984) with permission from Elsevier.

Note that the very act of making the measurement will solubilize some of the material. To correct for this error, simply measure and correct for the amount of protein in the supernatant after centrifugation. This additional measurement actually provides a "qualitative" solubility measurement for the material under study under the conditions of the experiment.

As the water uptake ability is partly structural, it is dependent on particle size, and thus the blending process must be standardized. For this purpose, the author has found the Omnimixer (Dupont's Sorvall Division) to be a particularly useful device. It is an "upside-down" blender that, most importantly, with the appropriate adapter, can be used to blend samples in centrifuge tubes, avoiding the need for quantitative transfer of some very sticky and viscous materials. Again, all the details of the method need to be standardized, even if this is done in an arbitrary fashion.

Although the conditions used for the measurement of both expressible moisture and water uptake ability are arbitrary, the two can be performed under similar conditions. One of the real benefits of the two tests, with arbitrarily defined working parameters, is that they can, in theory, be done in parallel, e.g., by placing four samples for expressible moisture and four samples for water uptake ability in the same eight-cell centrifuge. In this case, the differences observed reflect the differences between the two tests. This is illustrated in Figure B5.3.1.

Drip loss, cook loss, and thaw loss

Drip loss, cooking or processing loss, and thaw loss are similar to expressible moisture. In most cases, however, the moisture is lost without the need to apply a force. Thus, the liquid that is expelled can simply be collected by carefully "pouring off" the excess liquid. The drip loss test is a very quick and easy test that can be used with many samples. Drip loss measurements are slower than expressible moisture or water uptake ability measurements because they must include time for significant losses to occur.

It is important that any liquid be freely able to leave the sample. This is ensured either by controlling the geometry of the sample or by using other mechanical means (e.g., a screen) to ensure that channels are available for the liquid to drain into the collecting cylinder. Placing the sample in a funnel and collecting the drip in a graduated cylinder is often the easiest way to accomplish this. When cooking samples, the samples are often placed in a bag that is placed into boiling water. The drip is collected and a short period of time is allowed to collect further drip. In many cases, e.g., more "open" cooking methods, the weight loss of the actual sample may be more accurate than the collection of drip. Obviously, the loss of weight

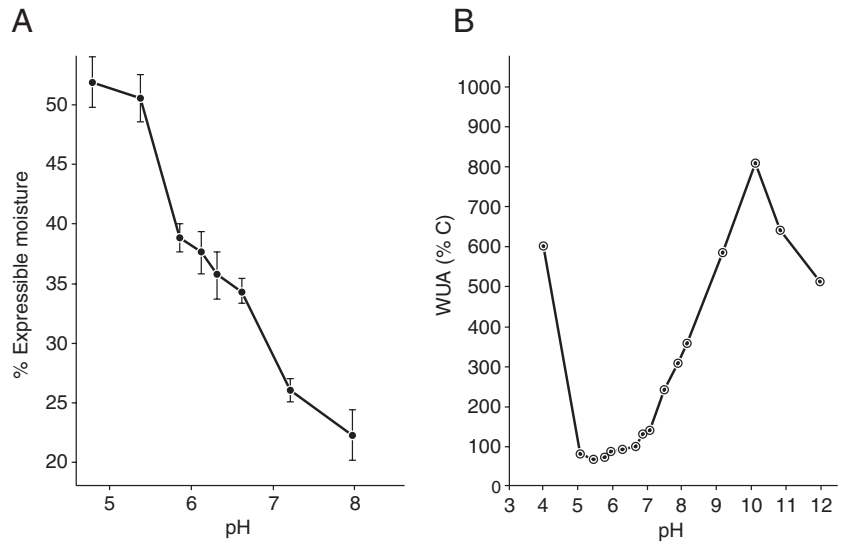

Figure B5.3.3 A comparison between expressible moisture and water uptake ability measurements as a function of pH. Reprinted from Regenstein (1984) with permission from the American Meat Science Association, and from Regenstein and Regenstein (1984) with permission from Elsevier.

of a product after cooking affects the perception of the food by the consumer, both because drip is generally undesirable and because weight loss makes a product reduce both in weight and volume.

Critical Parameters

Samples. For all tests described in this unit, the samples used must be representative of the material under study and should not be permitted to drip or dry out between sampling and actual experimentation. Samples must be han-

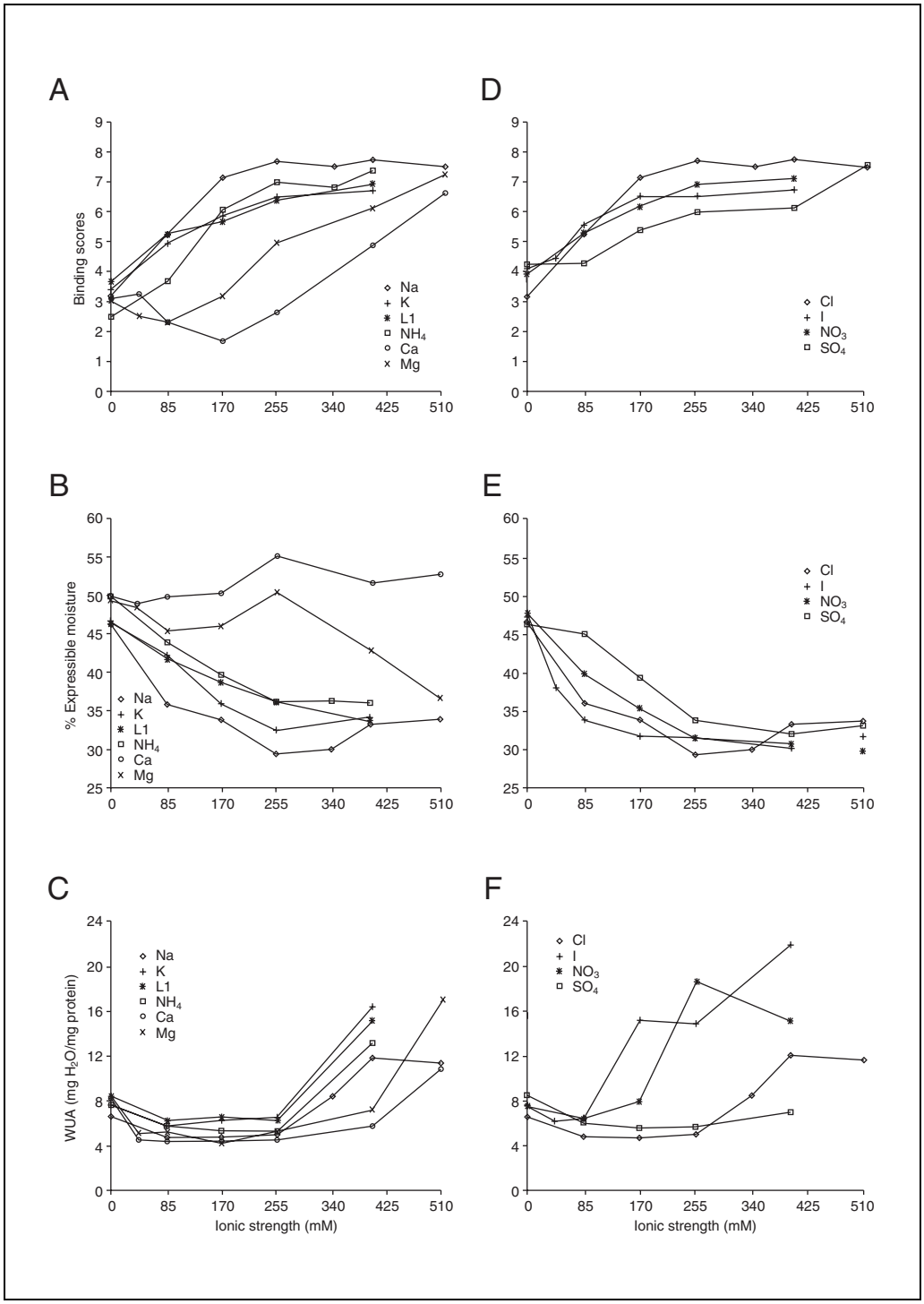

Figure B5.3.4 A comparison between moisture retention for a raw sample and the binding as measured by a sensory panel for fish muscle treated with different cations. Reprinted from Regenstein (1984) with permission from the American Meat Science Association and Zwika Weinberg (Volcani Center, Agricultural Research Organization, Israel).

dled carefully so that liquids are not expressed by the operator instead of the centrifuge.

Blending. Blending time for WUA must be carefully controlled, or can be used as an experimental variable. Note, however, that excessive blending may lead to emulsification, which would most likely lead to a poorer separation between the pellet and the supernatant. The amount of solution added must be carefully measured, as must the amount of supernatant after centrifugation.

Sample dilution. The solution used to dilute the sample needs to be carefully considered in terms of the objectives of the experiment. Remember that the sample itself will contribute some salt and will be at a particular pH.

Centrifugation. If samples for EM become impregnated into the filter paper despite the presence of the polyester mesh, the centrifuge speed must be decreased so that partitioning after centrifugation can be accurately done. For WUA, the removal of the supernatant layer (and any oil or denatured protein interface layer) must be done carefully. If the interface between precipitate and supernatant is not clear, the system must be carefully standardized, and higher centrifugation speeds should be considered. For WUA, higher centrifuge speeds are probably desirable, while for EM, lower speeds are desirable. In using such modifications, however, one loses the ability to perform the two tests under similar conditions.

Anticipated Results

Expressible moisture and water uptake ability measure different properties. Figure B5.3.2 shows the very different cation and anion dependencies of these methods using fish samples. Figure B5.3.3 shows that the pH profiles are also different. It is also apparent that WUA is often >100%, while expressible moisture must, of necessity, be <100%. Figure B5.3.4 shows an example where expressible moisture was actually correlated with a separate and independent functional measurement. In this case, the binding of cooked fish muscle as determined by a subjective sensory panel pulling samples of fish apparently paralleled the moisture retention of the raw fish (moisture retention = 1 – expressible moisture). Ideally, functional properties should show such correlations with other properties of interest in food systems.

Time Considerations

Measurement of expressible moisture and water uptake ability both require <1 hr. Drip loss takes 24 hr. Measurement of the moisture loss of samples requires 24 hr in a drying oven, plus cooling time in a desiccator. Actual set-up time is fairly short.

Protein determination by the Kjeldahl method is slow and very few samples can be run at one time. The Lowry and many other protein tests are much more convenient. A Lowry assay on a test tube rack of 40 samples can be done in <2 hr.

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Key References

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Presents the method for measuring expressible moisture.

Regenstein, 1984. See above.

A summary of the early results using the various methods discussed in this unit.

Regenstein, J.M., Gorimar, T.S., and Sherbon, J.W. 1979. Measuring the water holding capacity of natural actomyosin from chicken breast muscle in the presence of pyrophosphates and divalent cations. *J. Food Biochem.* 4:205-211.

Presents the method for measuring water uptake ability.

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Expression and Measurement of Enzyme Activity

The presence or absence of an enzyme is typically determined by observing the rate of the reaction(s) it catalyzes. Quantitative enzyme assays are designed to measure either the total amount of a particular enzyme (or class of enzymes) in units of moles or, more commonly, the catalytic activity associated with a particular enzyme. The two types of assays differ in that those in the latter category measure only active enzyme. The assays contained in this section are concerned primarily with the measurement of catalytic activity, or active enzyme. The assays are based on kinetic experiments, as activities are calculated from measured reaction rates under defined conditions. The basic Premise for these assays is that the amount of enzyme in a reaction mixture can be determined from the rate at which the enzyme-catalyzed reaction occurs.

ACTIVITY UNITS

The goal of most enzyme assays is to quantitatively measure the amount of enzyme activity (catalytic activity) present in a sample. Thus, assay results are typically reported in "activity" units. A unit of activity may be defined in various ways, but all such units are ultimately based on rates of substrate consumption and/or product formation. The most common units for expressing catalytic activity are the International Unit (also called the Unit or Enzyme Unit; U) and the Katal (Kat). The International Union of Biochemistry (IUB) originally defined a standard unit of enzyme activity (1 U) as that amount of enzyme that catalyzes the formation of 1 μmol product (or the conversion of 1 μmol substrate) per minute under standard conditions. In 1979, the Nomenclature Committee of the IUB recommended the use of the Katal as the fundamental unit of enzyme activity. The Katal is defined as that amount of enzyme that catalyzes the formation of 1 mol product (or the conversion of 1 mol substrate) per second under defined conditions. Thus, 1 Kat is equivalent to 6×10^7 U. The Katal was recommended because it is consistent with Système International (SI Units).

It is also common for enzyme activities to be reported in units based on changes in reaction mixture properties that are themselves a function of the extent of the enzymatic reaction. These units are often difficult to interpret in

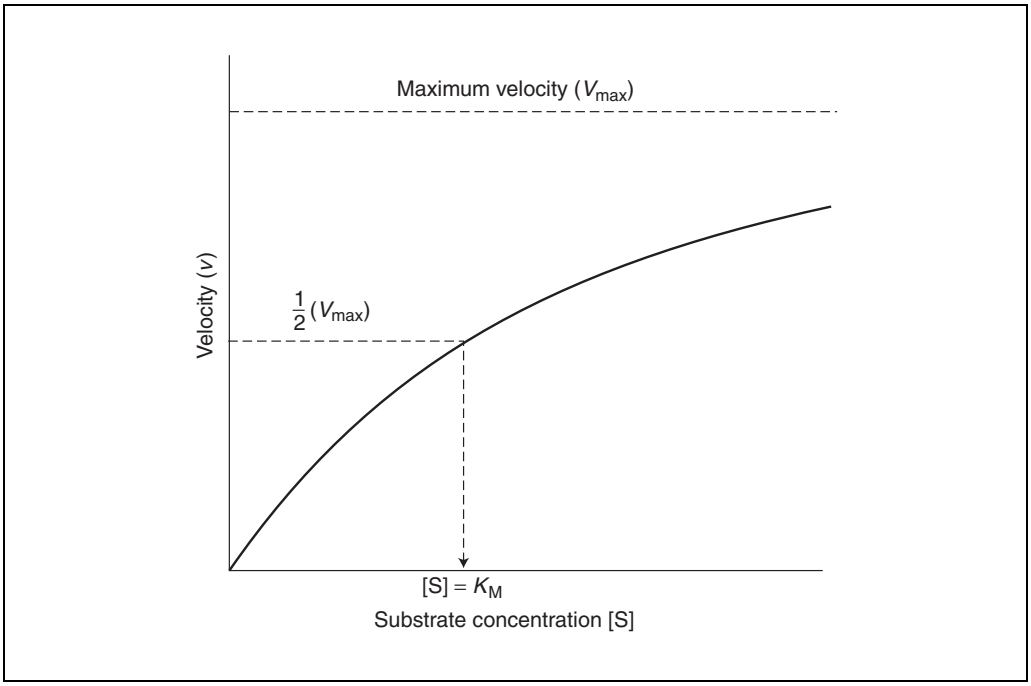

Figure C1.1.1 Relationship between substrate concentration and initial velocity at fixed enzyme concentrations. The Michaelis constant, K_M, is equal to the substrate concentration corresponding to one-half V_{max}.

Contributed by Michael H. Penner

Current Protocols in Food Analytical Chemistry (2001) C1.1.1-C1.1.4

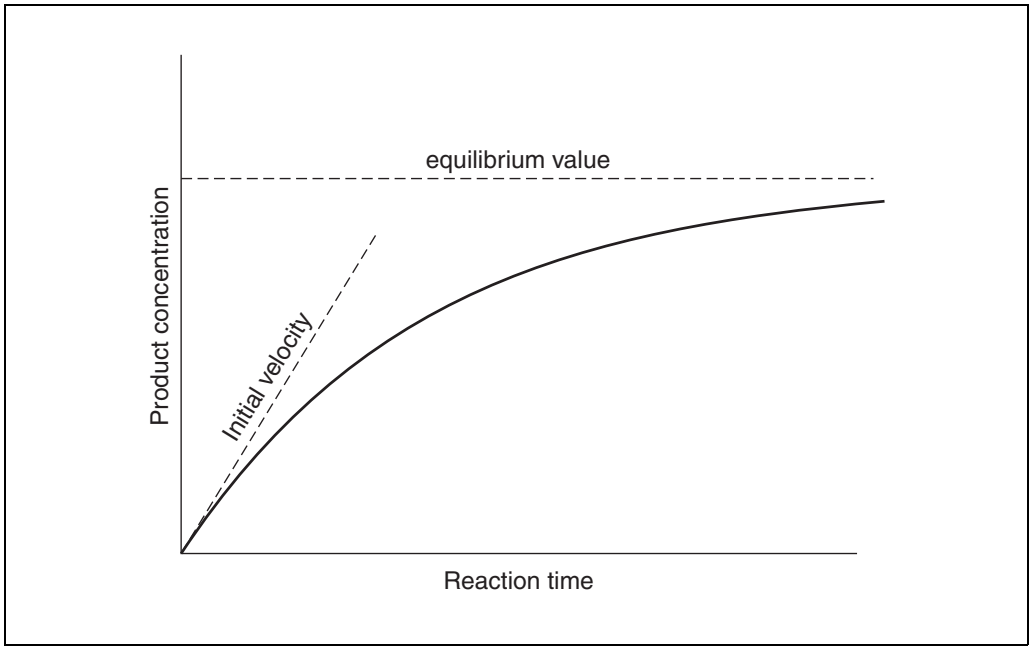

Figure C1.1.2 Time course of product generation for typical enzyme-catalyzed reaction. Product concentration is shown to asymptotically approach its equilibrium value (horizontal dashed line). The diagonal dashed line illustrates the portion of the curve used to calculate initial velocity.

terms of absolute numbers of catalytic events, but they can be used to effectively communicate relative amounts of enzyme activity. Assays of this type are particularly common when complex substrates and/or heterogeneous reaction mixtures are used. Changes in solubility, turbidity, and viscosity, per unit time, are examples of such units.

The upshot of the above discussion is that a variety of enzyme activity units may be encountered. This makes it essential that all units, whatever their bases, be clearly defined.

ASSAY CONDITIONS

Enzyme assays are typically done under relevant conditions, be they physiological conditions, food-storage conditions, or conditions corresponding to maximal activity. This implies that consideration must be given to reaction mixture parameters such as pH, temperature, ionic strength, buffer composition, and other components not involved in the reaction. It is prudent to assume that changes in any of these parameters may affect enzyme activity. Analysts will often run assays under apparent optimum conditions (maximal activity), such as optimum pH, because these conditions tend to coincide with maximum assay sensitivity. It should be apparent from this discussion that assays using different reaction conditions may have only limited comparative value.

Substrate concentration is yet another variable that must be clearly defined. The hyperbolic relationship between substrate concentration ([S]) and reaction velocity, for simple enzyme-based systems, is well known (Figure C1.1.1). At very low substrate concentrations ($[S] << K_M$), there is a linear first-order dependence of reaction velocity on substrate concentration. At very high substrate concentrations ($[S] >> K_M$), the reaction velocity is essentially independent of substrate concentration. Reaction velocities at intermediate substrate concentrations ($[S] \sim K_M$) are mixed-order with respect to the concentration of substrate. If an assay is based on initial velocity measurements, then the defined substrate concentration may fall within any of these ranges and still provide a quantitative estimate of total enzyme activity (see Equation C1.1.5). The essential point is that a single substrate concentration must be used for all calibration and test-sample assays. In most cases, assays are designed such that $[S] >> K_M$, where small deviations in substrate concentration will have a minimal effect on reaction rate, and where accurate initial velocity measurements are typically easier to obtain.

The composition of assay reaction mixtures is generally defined to the extent that it is practical. In most cases, the major source of unknowns is the enzyme preparation itself. Kinetically relevant compounds endogenous to

the enzyme source, such as substrates, inhibitors, and activators, may partition with the enzyme during sample preparation. Hence, for comparative purposes, it is recommended that enzyme preparation schemes be standardized.

INITIAL VELOCITY EXPERIMENTS

It is generally assumed that reported enzyme activities are based on initial velocity experiments, and that reported activities are proportional to the amount of active enzyme in the reaction mixture. Analysts should independently verify these assumptions in their laboratories, using the experimental systems particular to their situations. Initial velocity (or initial rate) kinetics implies that one is measuring the instantaneous velocity at the substrate concentration corresponding to the initiation of the reaction. Realistically, initial velocity often means the velocity that is measured as close to the initiation of the reaction as possible. Initial velocities are typically determined by collecting data points immediately after the initiation of the reaction, analyzing the data to confirm that the initial rate was actually observed (this may be as simple as showing that early data points define a linear rate of product formation), and then calculating the corresponding velocity in appropriate units. If the reaction is such that it is not experimentally feasible to obtain an initial linear rate of product formation, then a nonlinear regression technique can be used to estimate the initial velocity from the early phase of the reaction's time course.

Figure C1.1.2 shows a typical time course resulting from a continuous assay of product formation in an enzyme-catalyzed reaction. The hyperbolic nature of the curve illustrates that the reaction rate decreases as the reaction nears completion. The reaction rate, at any given time, is the slope of the line tangent to the curve at the point corresponding to the time of interest. Reaction rates decrease as reactions progress for several reasons, including substrate depletion, reactant concentrations approaching equilibrium values (i.e., the reverse reaction becomes relevant), product inhibition, enzyme inactivation, and/or a change in reaction conditions (e.g., pH as the reaction proceeds). With respect to each of these reasons, their effects will be at a minimum in the initial phase of the reaction—i.e., under conditions corresponding to initial velocity measurements. Hence, the interpretation of initial velocity data is relatively simple and thus widely used in enzyme-related assays.

ENZYME CONCENTRATION AND REACTION RATE

Enzyme assays are typically designed with the presumption that measured activities will be directly proportional to the amount of active enzyme in the reaction mixtures. Thus, the conceptual basis for most assays is that a linear relationship exists between measured activity and catalytic potential (active enzyme). This simple relationship is unlikely to be valid for all experimental permutations, and thus tests verifying the relationship between measured activity and quantity of enzyme should be included in all assays. This can be accomplished in a relatively easy way by assaying a series of samples that cover a range of enzyme concentrations and, based on the data, preparing a calibration curve of measured activity versus (relative) enzyme concentration, while making sure that enzyme concentrations in actual test samples fall within this range. For reference, enzyme concentrations in traditional assays are typically in the nanomolar to micromolar range.

A single-substrate reaction can be depicted as

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

Equation C1.1.1

where E is enzyme; S is substrate; ES is enzyme-substrate complex; P is product; and k_1, k_2, and k_3 are rate constants. The kinetics of such a unireactant enzyme system are most often described by the basic equation of enzyme kinetics, the Michaelis-Menten equation.

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

Equation C1.1.2

It expresses the velocity (v) of a single-substrate reaction (Equation C1.1.1) in terms of substrate concentration at time zero ([S]) and the kinetic constants K_M and V_{max}. V_{max} is defined as the limiting maximal velocity for the reaction, which is observed when all of the enzyme is present as ES. K_M, known as the Michaelis constant, is a pseudoequilibrium constant, which equals the concentration of substrate at which the reaction velocity equals one-half V_{max} (Figure C1.1.1).

V_{max} and K_M are defined by the following equations:

$$V_{max} = k_3 [E]_{Total}$$

Equation C1.1.3

$$K_M = \frac{k_2 + k_3}{k_1}$$

Equation C1.1.4

where $[E]_{total} = [E] + [S]$.

The velocity term, *v*, in Equation C1.1.2 refers to measured initial velocities. The equation's derivation is based on the assumptions that enzyme concentrations are much less than substrate concentrations ($[E]_{total} << [S]$) and that, over the course of the assay, the concentration of the enzyme-substrate complex remains essentially constant (the steady-state assumption). The point of this discussion is to show that measured initial velocities are expected to be directly proportional to the amount of active enzyme in reaction mixtures. Equation C1.1.5, obtained by substituting and rearranging the equations above, more clearly illustrates this relationship.

$$v = \left[\frac{k_3 [S]}{K_M + [S]} \right] [E]_0$$

Equation C1.1.5

In this equation, note that bracketed terms are constants under initial velocity conditions.

A nonlinear relationship between enzyme concentration and measured activity is indicative of a more complex reaction system. Complications of this nature may arise from such things as changes in the composition of the reaction mixture (e.g., pH due to the addition of increasing amounts of enzyme solution), assay limitations (e.g., insufficient substrate), limited coupling-enzyme (where assays are based on coupled enzyme systems), the presence of inhibitors, and enzyme-cofactor or enzyme-enzyme dissociation phenomena. Nonlinear relationships may also be an inherent outcome of assays employing complex substrates, such as starch granules, cellulose, and protein networks. In all cases it is advisable to verify the nature of the relationship between measured activity and quantity of enzyme (as discussed earlier). If the relationship is found to be nonlinear, then it is prudent to carefully check the assay to make sure that the nonlinear relationship is not simply an artifact of the experimental method.

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A good source of information on the design and execution of enzyme assays. The initial chapter, by K.F. Tipton, provides an excellent discussion of the general principles involved in enzyme assays. Subsequent chapters deal with specific assay approaches.

Segel, I.H. 1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons, New York.

A detailed, yet readable, discussion of rapid equilibrium and steady-state kinetics. The initial portion of the book deals with basic enzyme biochemistry, the kinetics of simple unireactant enzymes, and simple inhibition systems.

Whitaker, J.R. 1994. Principles of Enzymology for the Food Sciences, 2nd ed. Marcel Dekker, New York.

A classic text used for nearly 30 years to teach undergraduate and graduate students in the food sciences the fundamental principles of enzymology. The latter part of the text emphasizes the biochemistry of enzymes of particular relevance to the food sciences.

Wong, D.W.S. 1995. Food Enzymes: Structure and Mechanism. Chapman and Hall, New York.

This book gives an informative, yet relatively concise and well-referenced, summary of the structure and catalytic mechanism of selected food-related enzymes. All enzymes covered play an important role in food systems and all have their catalytic mechanism described at the molecular level.

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Detecting Enzyme Activity: A Case Study of Polygalacturonase

Polygalacturonase (PGase) is the trivial name commonly used in reference to poly-α-1,4-galacturonide glycanohydrolases (EC 3.2.1.15). These enzymes are glycosyl hydrolases, splitting the 1,4-glycosidic linkage between galacturonic acid residues in their linear polygalacturonic acid substrate. PGases can be either endo- or exo-acting. The endo-acting enzymes—those that are capable of catalyzing the hydrolysis of linkages in the interior of the polysaccharide chain—appear to be the most common.

The PGase enzymes are most commonly assayed using either reducing sugar– or viscosity-based methods. The former is based on the generation of a new reducing sugar with each catalytic event (Basic Protocol 1). The latter is based on the decrease in viscosity that occurs as a result of the enzyme-catalyzed hydrolytic degradation of polygalacturonic acid (Basic Protocol 2). The Support Protocol describes a method for isolating PGase from tomatoes.

STRATEGIC PLANNING

The two methods described herein are inherently different in that one is a traditional initial velocity assay that attempts to quantitatively measure rates of product formation (see Basic Protocol 1), whereas the other correlates the activity of an enzyme preparation with its ability to change the rheological properties (i.e., viscosity) of a substrate solution (see Basic Protocol 2). For both assays, it is presumed that the analyst is using soluble substrate and enzyme preparations, appropriate buffer systems, and a method to control the reaction mixture temperature. The ultimate goal of both assays is the same: to obtain a quantitative estimate of the PGase activity of a test solution.

Basic Protocol 1 makes use of the fact that one of the products resulting from the PGase-catalyzed reaction is a reducing sugar (Figure C1.2.1). Thus, in the simplest case, a determination of the number of reducing ends generated during a given reaction period will directly correspond to the number of catalytic events during that period. The assay requires a measurement of the number of reducing ends present at the initiation of the reaction and at a sufficient number of time points to establish an initial velocity. The assay is based on taking measurements at predetermined time points rather than continuously monitoring the extent of the reaction, because of the relatively harsh conditions necessary for the detection of reducing sugars. The assay is equally sensitive to endo- and exo-acting PGases, because both enzymes generate a single reducing end per catalytic event.

Basic Protocol 2 is based on the viscosity change that occurs in a polygalacturonic acid–containing solution as a result of enzyme activity. The assay does not directly measure the number of catalytic events, as is attempted in Basic Protocol 1, but rather a physical parameter that is a function of the number of catalytic events. Viscometric assays are typically done using capillary viscometers of the Ostwald type at a fixed predetermined temperature. Units for these assays are reported either as a function of the time required to reduce the viscosity of a given solution by a fixed percentage (Bateman, 1963) or as that amount of enzyme that will reduce the viscosity of the given solution by a fixed percentage in a fixed amount of time (Tagawa and Kaji, 1988).

The substrate prescribed for these assays is commercially available polygalacturonic acid. It was chosen based on its availability and on the observation that PGase enzymes are typically specific for glycosidic linkages between de-esterified galacturonic acid units

Contributed by Sasithorn Kongruang and Michael H. Penner
Current Protocols in Food Analytical Chemistry (2003) C1.2.1-C1.2.14

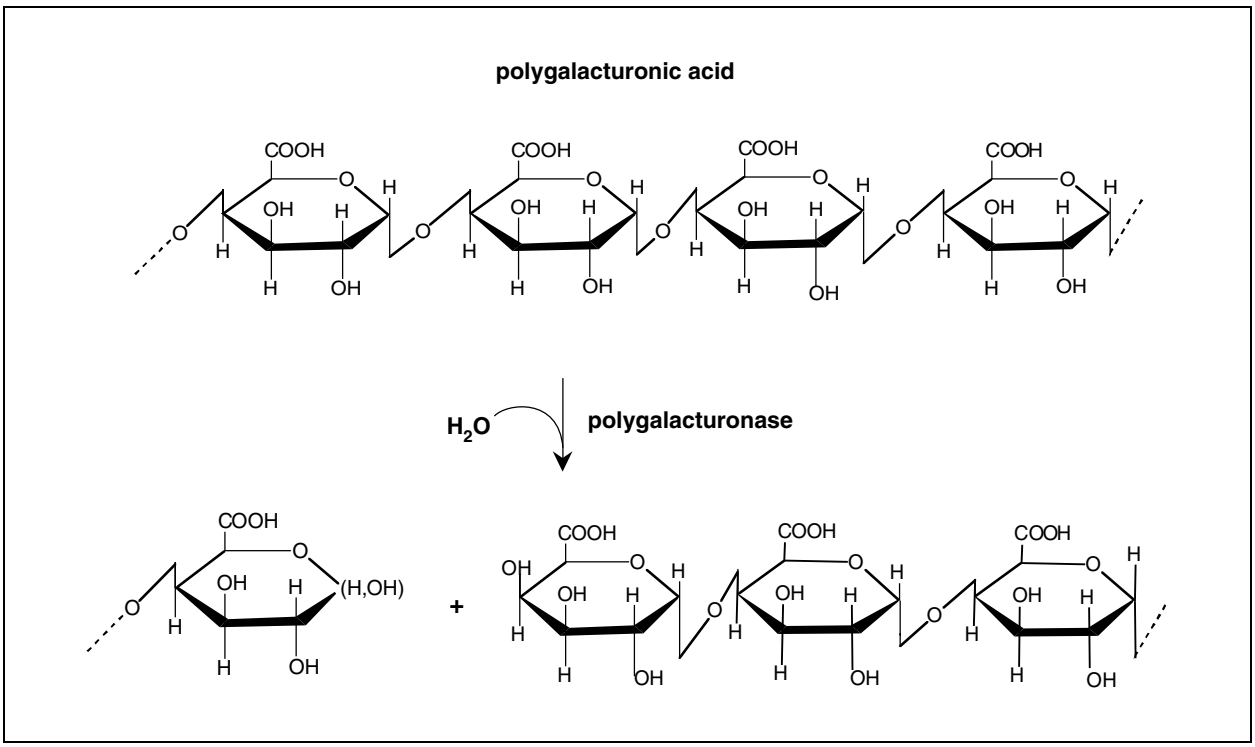

Figure C1.2.1 Reaction catalyzed by polygalacturonase.

(Schols and Voragen, 2002). Other substrates, such as citrus pectin, may be used in some cases, but overall PGase activities will probably be lower on the esterified pectins, and results from assays using the esterified pectins are more likely to be influenced by non-PGase enzymes, particularly pectin lyase and pectin methyl esterase (see Commentary).

The reaction conditions chosen for the assays are based on published optimal conditions for PGase enzymes. These enzymes typically have maximal activities at slightly acidic pH (Tucker and Seymour, 2002) and, in general, appear to be relatively stable at temperatures from 30° to 40°C. Optimal reaction conditions are likely to be enzyme specific, so one may have to alter the conditions to match the properties of the enzyme of interest. In all cases, the analyst should take into account the properties of the substrate, particularly its solubility, as well as the properties of the enzyme. For example, because solutions of polygalacturonic acid tend to gel as the pH is lowered below 3, viscometric assays (Basic Protocol 2) at these relatively low pHs are often not feasible.

The PGase extraction scheme outlined below (see Support Protocol) is based on the properties of the enzyme from tomato (Pressey, 1986). Enzymes from other sources will probably require different extraction conditions. A more detailed discussion of common approaches to enzyme extraction is included (see Background Information, discussion of samples for pectic enzyme assays).

DETERMINATION OF POLYGALACTURONASE ACTIVITY USING 2,2′-BICINCHONINIC ACID

Polygalacturonase (PGase) is most commonly assayed by measuring the liberation of reducing groups in reaction mixtures containing the enzyme and a suitable polygalacturonic acid substrate. The reaction conditions used in such assays correspond to those that are optimal for the enzyme, those that are pertinent to a particular physiological or food system, or (for comparative purposes) those that were previously established. In this assay, the authors recommend conditions that are in the functional range of typical PGases.

There are several colorimetric assay reagents available for measuring the number of reducing groups generated over the course of the PGase reaction, including Nelson copper-arsenomolybdate (Nelson, 1944; Tagawa and Kaji, 1988), 2-cyanoacetamide (Honda et al., 1980; Gross, 1982; Bach and Schollmeyer, 1992), 3-methyl-2-benzothiazolinone hydrozone (MBTH; Anthon and Barrett, 2002), 2,2′-bicinchoninate (BCA; Doner and Irwin, 1992; Garcia et al., 1993), modified dinitrosalicylic acid (Wang et al., 1997), and 4-hydroxybenzoic acid hydrazide (Lever, 1972; Atkinson et al., 2002). The assay presented here uses the 2,2′-bicinchoninate acid method to quantify reducing ends.

The assay is presented as if a single unknown enzyme preparation is being tested for PGase activity. The assay includes the determination of reaction rates at two enzyme loads (plus corresponding enzyme and substrate blanks) to verify that the measured activity is proportional to the amount of enzyme in the reaction mixture. It suggests measuring four time points per enzyme load to ascertain appropriate initial velocities. The assay does not indicate the number of replicates for each measurement, because this is partially dictated by familiarity with the assay. The authors suggest doing all assays in either duplicate or triplicate.

The assay contains both enzyme and substrate blanks. For the purpose of this unit, the enzyme blank contains all reaction mixture components except the enzyme preparation, and the substrate blank contains all reaction mixture components except the substrate. The substrate blanks and enzyme blank are used to account for any apparent rates of reaction that are attributable to factors other than the specific interaction of the PGase enzyme with the polygalacturonic acid substrate.

Materials

0.25% (w/v) polygalacturonic acid solution (see recipe)
20 mM sodium acetate, pH 5.0 (*APPENDIX 2A*)
BCA working reagent (see recipe)
Enzyme preparation (see Strategic Planning and see Support Protocol)
1 mM galacturonic acid (see recipe)

12 × 75–mm glass test tubes
37° and 80°C water baths

Set up reaction tubes

1. Label five 12 × 75–mm glass test tubes as follows:

   Enzyme load 1 and 2
   Substrate blank 1 and 2
   Enzyme blank.

   If checking the PGase activity associated with a single enzyme preparation, there will be five test solutions. The enzyme load and substrate blanks are done at two different enzyme concentrations. With duplicate or triplicate analyses, there will thus be ten or fifteen tubes in total.

2. To each of the three substrate-containing tubes (enzyme load and enzyme blank), add 0.2 ml of 0.25% polygalacturonic acid solution and the appropriate amount of 20 mM sodium acetate such that the final volume of the reaction mixture will be 1.0 ml following the addition of the enzyme preparation (step 6).

3. To the two substrate blank tubes, add the appropriate amount of 20 mM sodium acetate such that the final volume of the reaction mixture will be 1.0 ml following the addition of enzyme preparation (step 6).

4. Place all tubes in a 37°C water bath and allow time for them to equilibrate to that temperature.

Initiate reaction and collect time points

5. Set up a series of four 12 × 75–mm glass test tubes for each of the five test solutions described above. Label tubes within each set to correspond to an appropriate time point (e.g., 0, 30, 60, and 120 min). Add 1 ml cold BCA working reagent to each tube and place tubes on ice.

   These tubes will be used for termination of the reaction and for color development of the reducing sugar.

6. Initiate the reaction in the enzyme-containing tubes (all tubes except enzyme blank) by adding the appropriate amount of enzyme preparation with gentle mixing. Maintain reaction mixture at 37°C throughout the reaction period.

   The amount of enzyme preparation added will depend on the relative activity of the enzyme preparation. A convenient starting point would be to add 40 µl enzyme preparation to enzyme load 1 and substrate blank 1, and 80 µl to enzyme load 2 and substrate blank 2.

7. At the specified times (e.g., 0, 30, 60, and 120 min), remove 0.1 ml reaction mixture (or blank solution) and add it to its corresponding termination tube containing BCA reagent (step 5). Terminate the 0 time point immediately after the reaction is initiated and place on ice. Proceed until the final time points are collected.

   The high pH of the resulting solution, combined with the relatively low temperature, will effectively terminate the reaction.

8. Include with the five sets of termination tubes a series of five tubes to be used to establish the calibration curve. Place in the calibration tubes 0, 5, 10, 15, and 20 µl of 1 mM galacturonic acid. Then add 20 mM sodium acetate to bring the volume to 0.1 ml and add 1 ml BCA working reagent (final volume 1.1 ml/tube).

   The standards correspond to 0, 5, 10, 15, and 20 nmol galacturonic acid. They can be set up in duplicate or triplicate, depending on the experience of the analyst.

Perform color development

9. Transfer all BCA-containing reaction mixtures to an 80°C water bath to initiate color development. Cover each tube with a glass marble, Parafilm, or other suitable covering to minimize evaporation, and leave tubes in bath for 30 min.

10. Transfer tubes to cold water for 10 min and centrifuge 5 min at 14,000 × *g*, room temperature, to clarify solutions.

11. Read absorbance with a spectrophotometer set at 560 nm using water as the reference.

Calculate results

12. For each test solution, plot time versus absorbance and obtain the slope (i.e., the reaction rate) by linear regression.

13. Correct the rate of reaction for the enzyme/substrate solution by subtracting the values obtained for the corresponding substrate and enzyme blanks.

14. To generate a calibration curve, use data from the galacturonic acid–containing tubes (step 8) and plot moles galacturonic acid versus absorbance.

15. Convert absorbance values to moles galacturonic acid equivalents using the calibration curve.

    The PGase activity of the unknown enzyme preparation, in terms of reducing groups generated per unit time, is based on the corrected rate of increase in galacturonic acid equivalents for the experimental enzyme/substrate test solutions. One unit of enzyme activity (katals) is defined as that amount of enzyme that liberates 1 mole of reducing sugar per second under the defined conditions.

VISCOSITY-BASED ASSAY FOR POLYGALACTURONASE ACTIVITY

BASIC PROTOCOL 2

Polygalacturonase (PGase) is an example of a polymer depolymerase enzyme, and the activity of this type of enzyme has been measured by viscosity-based methods for many years. These assays are based on the presumption that the viscosity of a polymer-containing solution is a function of the molecular weight of its component polymers. Thus, in this assay, the degradation of polygalacturonic acid, as catalyzed by PGase, results in a lowering of the viscosity of the polygalacturonic acid–containing solution. In this assay, the viscosity of the substrate solution is determined as a function of the efflux time required for the solution to pass between the indicated marks on an Ostwald viscometer. This assay has evolved for over forty years (Mill and Tuttobello, 1961; Bateman, 1963; Tagawa and Kaji, 1988; Gusakov et al., 2002); the one presented here is adapted from Tagawa and Kaji (1988) and Gusakov et al. (2002).

NOTE: This protocol assumes that the reader is familiar with capillary viscometer methodology. For a general introduction, see *UNIT H1.3*.

Materials

0.7% to 1.0% (w/v) sodium pectate in 20 mM sodium acetate, pH 5.0 (*APPENDIX 2A*)
Enzyme preparations (see Support Protocol), both active and heat inactivated, in 20 mM sodium acetate, pH 5.0

Ostwald viscometer (capillary diameter, 0.5 mm; e.g., Fisher Scientific)
Constant-temperature mechanism (e.g., appropriate water bath)

1. Pipet 5.0 ml of 0.7% to 1.0% sodium pectate solution into an Ostwald viscometer and bring to 30°C.

   At the optimal sodium pectate concentration, the efflux time of the substrate solution in the viscometer should be in the range of 115 to 120 sec.

2. Add 100 µl active enzyme preparation to the viscometer with mixing. Start a timer to monitor reaction time.

   Adding the enzyme initiates the degradation of polygalacturonic acid.

3. Determine an efflux time immediately after initiating the reaction (using a second timer) and then at suitable intervals to allow graphical determination of the time to reach a 50% reduction in viscosity.

   If the reaction proceeds too quickly, the enzyme preparation should be diluted with buffer, and the assay should be repeated. One should attempt to obtain at least two time points prior to there being a 50% reduction in the relative viscosity of the reaction mixture.

4. Set up a second reaction system as described above, but use a heat-inactivated enzyme. Use the efflux time of this reaction as the zero-time data point (V_0).

   In general, enzymes can be thermally inactivated by bringing the enzyme-containing solution to 95° to 100°C for 5 min. However, one should always verify that treatment results in complete inactivation (via a check for residual activity), since some enzymes are particularly heat stable.

5. Set up a third reaction system using heat-inactivated enzyme diluted in 20 mM sodium acetate buffer alone (no sodium pectate substrate solution). Use the efflux time of this reaction as V_s.

6. Determine the percentage fall in viscosity (A), which is defined as follows (Roboz et al., 1952):

$$A = \frac{V_0 - V_t}{V_0 - V_s} \times 100$$

   where V_0 is the efflux time of sodium pectate solution plus inactivated enzyme (step 4), V_t is the efflux time of sodium pectate solution plus enzyme (step 3), and V_s is the efflux time of inactivated enzyme in buffer alone (step 5). All efflux times are measured in seconds.

   A unit of activity is arbitrarily defined as that amount of enzyme that will reduce the viscosity of the sodium pectate solution by 50% in a 5-min period. The time required to reach A = 50% is typically inversely proportional to the concentration of enzyme (Mill and Tuttobello, 1961; Gusakov et al., 2002).

SUPPORT PROTOCOL

POLYGALACTURONASE ENZYME PREPARATION

In most food applications, the analyst is working with rather complex matrices. This raises the question of how to quantitatively extract a representative fraction of the enzyme to be assayed. In the best-case scenario, all or a representative fraction of the active target enzyme will be obtained in a solution devoid of other components that may hinder the assay (compounds that may affect either the enzyme itself or some other aspect of the assay). The objective of many assays is to measure the total amount of enzyme activity associated with a particular sample; this determination depends on the quantitative extraction of the target enzyme. In all cases, it is essential that the enzyme preparation be clearly defined when reporting the amount of enzyme associated with a given product, because this step is likely to have a major impact on measured activities. Furthermore, it should not be assumed that an enzyme preparation protocol optimized for a particular sample or product is necessarily optimal for a different sample.

Optimal conditions for the extraction of polygalacturonase (PGase) depend on the sample matrix (type of food product) and the source of the enzyme (plant versus microbial origin). The extraction scheme presented below is that developed for the PGase of tomato (Pressey, 1986).

Materials

Tomato, sliced
Water, 4°C
0.1 N HCl
1.2 M NaCl, pH 6.0
Homogenizer

Additional reagents and equipment for dialysis or size-exclusion chromatography

1. Add 100 g sliced tomato to 100 ml 4°C water and homogenize.
2. Adjust homogenate pH to 3.0 with 0.1 N HCl and leave to settle for 15 min at 4°C.
3. Centrifuge 20 min at 8,000 × *g*, 4°C, and discard supernatant.
4. Wash the pellet twice. Each time suspend the pellet in cold water (adjust to pH 3 with 0.1 N HCl, if necessary), centrifuge 20 min at 8,000 × *g*, and decant the supernatant.
5. Extract PGase from the washed pellet by suspending pellet in 100 ml of 1.2 M NaCl and centrifuging 20 min at 8,000 × *g*. Collect supernatant.
6. Remove salt while exchanging the reaction mixture with an appropriate buffer system by dialysis or size-exclusion chromatography.

   For the protocols described here, 20 mM sodium acetate buffer, pH 5.0, is appropriate.

   The molecular weights of PGases from different sources are expected to vary. For example, tomato PGase exists in two forms, PG1 and PG2, which have molecular weights of 84 and 44 kDa, respectively (Wong, 1995). Hence, one should not assume a molecular weight for novel polygalacturonases, this being important when choosing separation techniques based on size-exclusion principles.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCA (2,2′-bicinchoninic acid) working reagent

For assay solution A: Dissolve (in order) 12.1 g sodium bicarbonate ($NaHCO_3$), 27.4 g sodium carbonate (Na_2CO_3), and 971 mg BCA sodium salt in 450 ml distilled water with stirring. Bring to 500 ml with distilled water in a volumetric flask. Store in a dark bottle up to 1 month at room temperature.

For assay solution B: Dissolve 624 mg copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and 631 mg L-serine with stirring. Bring to 500 ml with distilled water in a volumetric flask. Store in a dark bottle up to 1 month at 4°C.

For working BCA reagent: On the day of the experiment mix equal volumes of assay solution A and assay solution B.

Galacturonic acid, 1 mM

Prepare stock 10 mM galacturonic acid solution by dissolving 0.2122 g D-galacturonic acid monohydrate in 100 ml of 20 mM sodium acetate buffer, pH 5.0 (*APPENDIX 2A*). Dilute this stock solution 10-fold with buffer. Store up to 1 week at 4°C.

Polygalacturonic acid solution, 0.25% (w/v)

Disperse 0.5 g polygalacturonic acid (Sigma) in 100 ml distilled water by stirring at room temperature in a 1-liter beaker. With continued stirring, add 400 ml of 95% (v/v) ethanol to precipitate the polygalacturonic acid (free galacturonic acid will remain in solution). Collect the precipitate by vacuum filtration of the wash suspension through Whatman no. 1 filter paper. Dry the ethanol-washed polygalacturonic acid at 22°C overnight.

Dissolve 0.25 g ethanol-washed polygalacturonic acid in 70 ml of 20 mM sodium acetate buffer, pH 5.0 (*APPENDIX 2A*). With continued stirring, adjust pH to 5.0 with 0.1 N NaOH, and then bring to a final volume of 100 ml with buffer. Divide into aliquots and store at 0°C for up to 2 months.

COMMENTARY

Background Information

Pectic polysaccharides

Pectins are a group of polysaccharides found in the primary cell walls and intercellular regions of higher plants (*UNITS E3.1-E3.4*); they are typically the most abundant polymers in these regions of fruit (Willats et al., 2001). They can be extracted with relatively mild aqueous solvents (such as mildly acidic or basic water and/or chelator-containing water). Their function appears to be to maintain the integrity and rigidity of cell walls, to enhance water retention, and to act as an adhesive between cells (Schols and Voragen, 2002). Polygalacturonase (PGase) is a hydrolytic enzyme that acts on pectic polysaccharides.

Pectic polysaccharides are heterogeneous with respect to chemical structure and size. The major components are polymers comprised primarily of α-D-galactopyranosyluronic acid building blocks. Linear polymers containing only these moieties, associated via covalent (1-4)-linkages, are termed homogalacturonans. Varying percentages of the individual building blocks in homogalacturonans are typically esterified at the 6-position carboxyl group with methanol. More complex pectic polysaccharides, having neutral sugar-containing branched segments interspersed along the linear homogalacturonan polymer, have also been obtained from several fruit tissues (Schols et al., 1990). The different pectin polymers are typically divided into several different groups: homogalacturonans, xylogalacturonans, rhamnogalacturonans, arabinans, arabinogalactans, and apiogalacturonans. The key feature for the major pectic polysaccharides is the presence of linear chain regions comprised of (1-4)-linked α-D-galactopyranosyluronic acid units (BeMiller, 1986).

Pectic enzymes

Pectic enzymes are produced by plants and microorganisms. The major depolymerase enzymes—that is, those that split the (a1-4)-glycosidic linkages in the backbone of the homogalacturonans—are PGase and pectic lyase (PL). The other major pectic enzyme is pectinesterase (PE). PE catalyzes the hydrolytic removal of methoxy groups from methylated pectic substances. PGase (EC 3.2.1.15) is a hydrolytic pectin depolymerase produced by both microbial and plant tissues. It catalyzes the hydrolysis of the (α1-4)-linkages between two adjacent galacturonic acid residues within the homogalacturonan backbone (Figure C1.2.1). Generally, PGases act at glycosidic linkages adjoining two de-esterified galacturonic acid residues. The enzymes normally have an optimal pH in the acid range (pH 4 to 6) and can be either exo- or endo-acting; endo-acting PGase is the most common (Tucker and Seymour, 2002).

PL (EC 4.2.2.2; sometimes called pectate transeliminase) is a pectin depolymerase like PGase, but it is believed to be produced only by fungi and bacteria, and not by higher plants. The PL-catalyzed reaction goes via a β-eliminative cleavage. The reaction generates a new reducing end and a new nonreducing end (Figure C1.2.2). The new nonreducing end differs from that produced in the PGase-catalyzed reaction in that the terminal sugar unit is a 4-deoxy-α-D-galacto-4-enuronosyl group. The PLs are typically subdivided based on their specificity for pectinic versus pectic acid and whether they are exo- or endo-acting. The optimal pH for the PLs is generally in the range of 8.5 to 9.0, and all appear to require Ca^{2+} for activity (Whitaker, 1994).

Pectic enzyme assays

Reducing end–based assays are widely used to measure pectin depolymerases. These assays take advantage of the fact that the primary depolymerase enzymes, PGase and PL, generate a new reducing end as a consequence of each catalytic event. Therefore, enzyme activity can be followed by monitoring the increase in reaction mixture reducing ends as the depolymerases act on the appropriate substrate, either pectic or pectinic acid. Quantification of reducing ends is typically done using a colorimetric assay under highly alkaline conditions. Hence, they are discontinuous assays in which the reaction is terminated and the number of reducing ends determined. There are several different colorimetric assays for quantifying reducing ends (Nelson, 1944; Gross, 1982; Doner and Irwin, 1992; Anthon and Barrett, 2002).

When working with pure PGase, the results of the reducing sugar–assay presented here will clearly reflect the inherent activity of that enzyme on the chosen substrate. In many cases, however, analysts are working with rather crude enzyme preparations that may contain a combination of PGases and PLs. In such cases, reducing sugar–based assays would be expected to reflect a combined PGase and PL

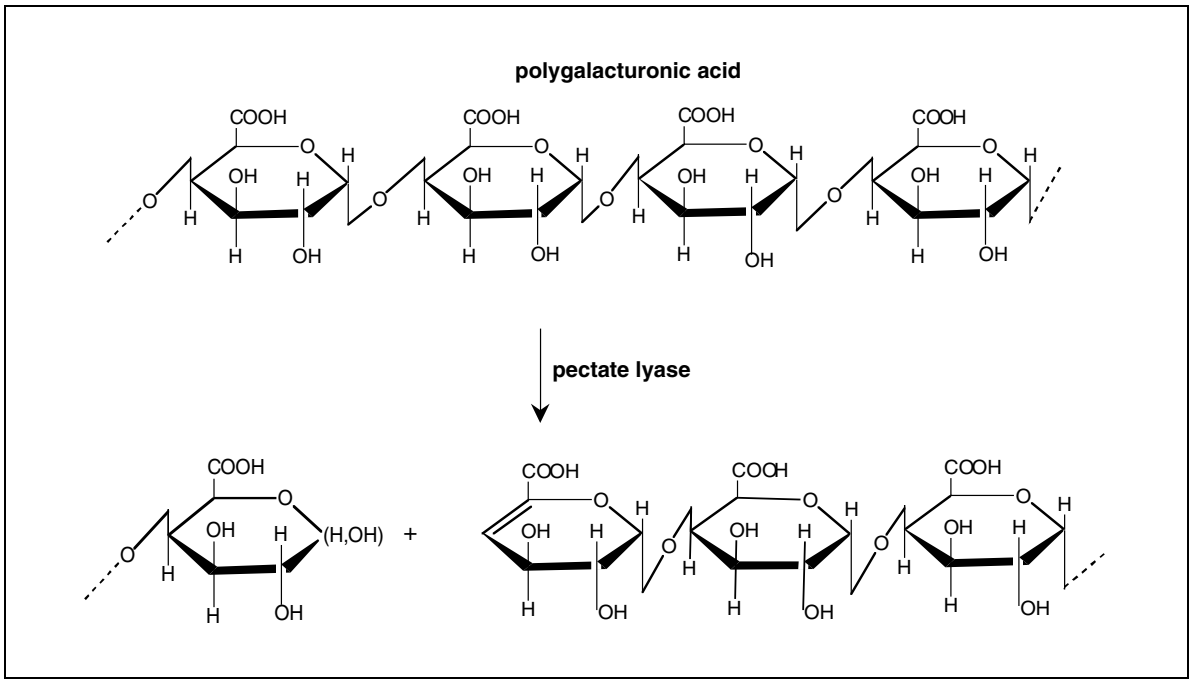

Figure C1.2.2 Reaction catalyzed by pectate lyase.

activity. Hence, if one wants to focus only on PGase, it is important to account for potential lyase activity. This is done by choosing reaction conditions that favor PGase activity, such as using a low methoxy pectin substrate (i.e., polygalacturonic acid), a relatively low reaction mixture pH, and a calcium chelator (to sequester Ca^{2+}). Even with these optimized conditions, however, it is possible that lyase activity is occurring. To check for this, a rather simple spectrophotometric assay can be used that is based on the fact that the lyase reaction yields, along with the new reducing end, a 4,5-unsaturated nonreducing end that absorbs radiation at 232 nm (Collmer et al., 1988).

PGase and PL, as stated above, are both depolymerases. Viscosity assays have been used to measure combined depolymerase activity for more than fifty years (Owens et al., 1946). The viscosity of pectin-containing solutions, as well as that of other polymer-containing solutions, is a function of the shape and molecular weight of the component polymers. Depolymerase activity is expected to lower the viscosity of a polymer-containing solution, provided that other parameters affecting viscosity remain constant, because the enzymatic activity results in a lowering of the polymer's molecular weight. The rate of reduction in the viscosity of a polymer-containing solution can thus be used to measure depolymerase activity. Viscosity-based assays are more sensitive to endo-acting depolymerases as compared with exo-acting depolymerases because, on average, there is a greater change in molecular weight per endo-depolymerase-catalyzed reaction.

Traditional viscosity assays use either a capillary or a rotating spindle viscometer (Sherwood and Kelman, 1964; Gusakov et al., 2002). The viscometric assay presented in Basic Protocol 2 is intended to measure PGase activity with minimal interference from PL. PGase activity is favored by using polygalacturonic acid as the substrate rather than pectins with higher methoxy content, by using a relatively low reaction mixture pH, and by avoiding the addition of Ca^{2+} to the reaction mixture. The authors recommend that PL activity be monitored, as discussed above (Collmer et al., 1988), at least during the initial phases of the analysis, to confirm that combined PGase and PL depolymerization is not measured.

Samples for pectic enzyme assays

In the Support Protocol, the tomato puree is first washed with water to remove water-soluble components, particularly reducing sugars, and is then extracted with an equal weight of 1.2 M NaCl. The procedure is appropriate for this application because the tomato enzyme is sparingly soluble in water at pH 3, but is soluble in relatively high salt solutions. The advantage of this procedure is that the water-soluble components can easily be removed prior to extracting the enzyme. This is particularly nice for the reducing sugar–based assay, because the sol-

uble reducing sugars inherent in the enzyme-containing sample add unwanted background signal in the final assay (i.e., they give a high blank absorbance reading).

The authors have applied this same procedure to peach purees, although at the pH of the peach product (~3.9). In that case, however, the PGase activity associated with the peach puree was relatively soluble in water. Hence, the initial water extracts had significant PGase activity. Therefore, one may choose to assay the water extract itself. As noted in the protocol for removal of salt, the soluble reducing sugars in the water extract can be removed by techniques such as dialysis or size-exclusion chromatography.

Difficulties in extracting enzyme from different sample matrices may result when the enzyme (1) is covalently or noncovalently associated with insoluble components, (2) is physically entrapped in the tissue matrix such that it is inaccessible to solvent, or (3) is itself insoluble in the chosen solvent. Several papers suggest that a percentage of the PGase inherent in the tomato is immobilized in the cell wall and thus is insoluble (Hobson, 1964; Pressey, 1986; Jackman et al., 1995). Researchers have developed two experimental approaches to getting this enzyme into solution. First, some of the immobilized or inextractable enzyme can be freed from the fruit by disrupting the tissue's ionic complexes (by adding high salt or increasing the pH of the extraction buffer to >6). The ionic complexes probably include negatively charged pectate polymers with cationic regions on the PGase enzyme. Second, some of the immobilized enzyme can be solubilized by disrupting the physical structure of the cell wall through chelation of Ca^{2+}.

It is not clear if the types of interactions that dictate the behavior of tomato PGase are common to other PGase food systems. Certainly, one may expect that microbial PGases (e.g., those associated with rotting fruit) will have somewhat different associations with the food matrix. Enzyme-tissue ionic complexes are probably important in the case of exogenous microbial PGase, but physical entrapment probably is not.

Critical Parameters

The assays for PGase activity presented here are based on measurements of reaction rates. Hence, all experimental parameters that may affect the rate of an enzyme-catalyzed reaction (including pH, ionic strength, buffer composition, and temperature) need to be defined. These parameters are typically chosen to coincide with optimal or biologically relevant conditions. The protocols presented here specify reaction conditions that are appropriate for typical PGases. An analyst may choose to change these conditions to better reflect the properties of a novel enzyme or to better simulate the conditions present in a particular food product.

The reducing sugar–based assay is intended to provide initial velocity kinetics. This means that the analyst must establish, for all experimental permutations, that initial rates are indeed being measured. These topics, as well as other information related to the design of enzyme assays, are discussed in *UNIT C1.1*.

The substrate chosen for the assays presented in this unit is polygalacturonic acid. As explained previously, use of this substrate is advisable because PGases typically have higher activity, and lyases lower activity, toward this low-methoxy substrate. An analyst may, however, want to use a more highly esterified substrate to simulate pectins of a particular product. If this is the case, the analyst must consider two potential complications. First, the PGase activity toward the more esterified substrate is probably sensitive to the presence of pectin methylesterase activity. This type of interaction is not a problem when working with pure enzymes, but it can be a problem when using crude enzyme preparations. Significant methylesterase activity will create regions of low methoxy content in the esterified substrates, and these low-methoxy regions are likely to be more susceptible to PGases. The net result of simultaneous methylesterase activity would be an increase in measured PGase activity. The second potential complication is that common reducing sugar assays make use of relatively high temperatures (80° to 100°C) and alkaline reaction mixtures (pH > 10). These conditions are conducive to the production of non-enzyme-derived reducing sugars via a base-catalyzed β-elimination reaction (Keijbets and Pilnik, 1974). This may lead to the production of non-polygalacturonidase-derived reducing sugars. This complication can be avoided by using Basic Protocol 2.

In some cases, particularly when working with samples containing low PGase activity, it may be necessary to run assays for extended reaction periods. One of the concerns with extended assays is the potential for microbial growth to interfere with assay results. This may be avoided by including an antimicrobial agent in the reaction mixture. The stipulation that

goes with this approach is that the antimicrobial agent must not significantly interfere with the assay. The authors have used thimerosal and sodium azide as antimicrobial agents in PGase assays. Thimerosal was initially chosen because it has been shown to be effective in other enzyme assays without having an adverse effect on enzyme activity. Because thimerosal reacts with the BCA reagent, however, an alternative reducing sugar assay, such as the 3-methyl-2-benzothiazolinone hydrazone (MBTH) procedure (Anthon and Barrett, 2002), must be employed. Sodium azide is another widely used antimicrobial agent and it does not appear to interfere with the BCA assay.

Anticipated Results

The results from Basic Protocol 1 are expected to be consistent with traditional initial velocity assumptions for enzyme kinetics (*UNIT C1.1*). The assay, as presented, includes four time points (along with a zero-time value) in order to establish the relationship between reaction time and product formed. Representative data, demonstrating the hyperbolic nature of this relationship, are presented in Figure C1.2.3. In this case, only the initial time points at the lowest enzyme concentration are consistent with the linear initial velocity assumption. If that relationship is required for estimating enzyme activity, then one may try to improve the estimates of initial velocity either by obtaining more data points earlier in the progress of the reaction or by diluting the enzyme preparation and assaying again over the same time frame. As will be seen in the following paragraph, a linear relationship between enzyme load and product generated per specified time is obtained in the present case without measurements of true initial velocities.

The assays presented here are based on there being a linear relationship between the amount of active enzyme present in a reaction mixture and the observed activity for that reaction mixture. To verify this, the analyst must assay a minimum of two enzyme concentrations (as suggested in Basic Protocol 1). The expected relationship is demonstrated in Figure C1.2.4. In this example, four enzyme loadings were assayed and, as expected, the measured activity is proportional to the amount of enzyme present.

The reducing sugar–based assays are predicated on the correct measurement of enzyme-generated product. A typical calibration curve for the BCA assay, using galacturonic acid as the calibration standard, is presented in Figure C1.2.5. Note that the assay is linear for the

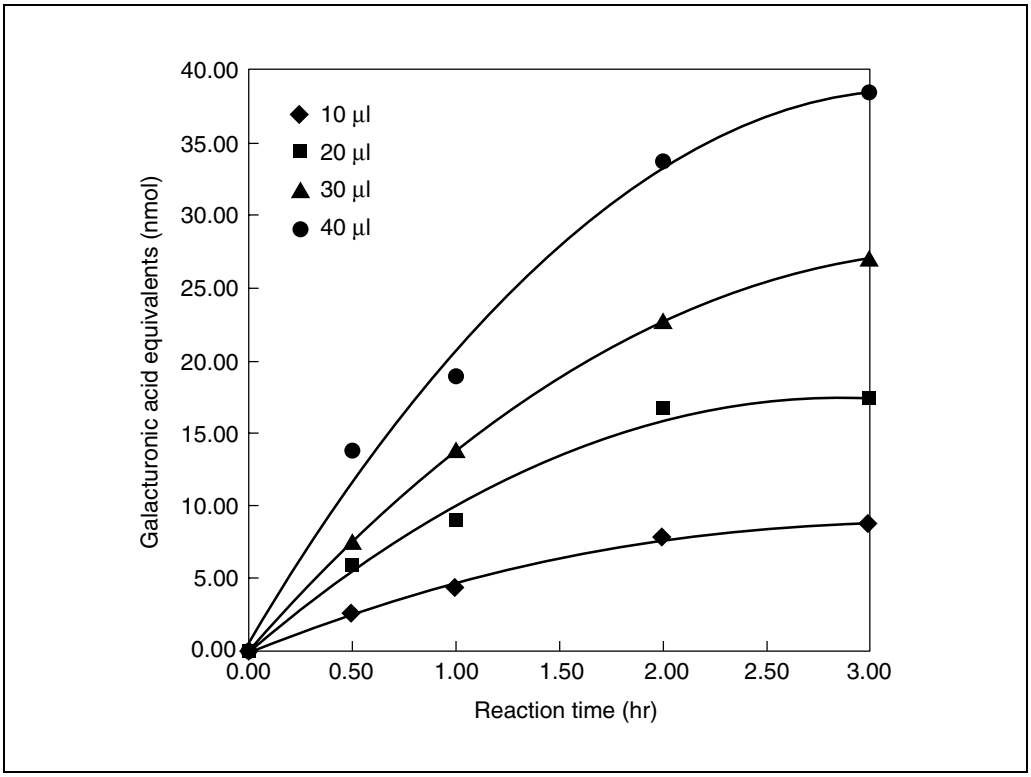

Figure C1.2.3 Reaction progress curves for the production of new reducing ends (measured as galacturonic acid equivalents) at different enzyme loads.

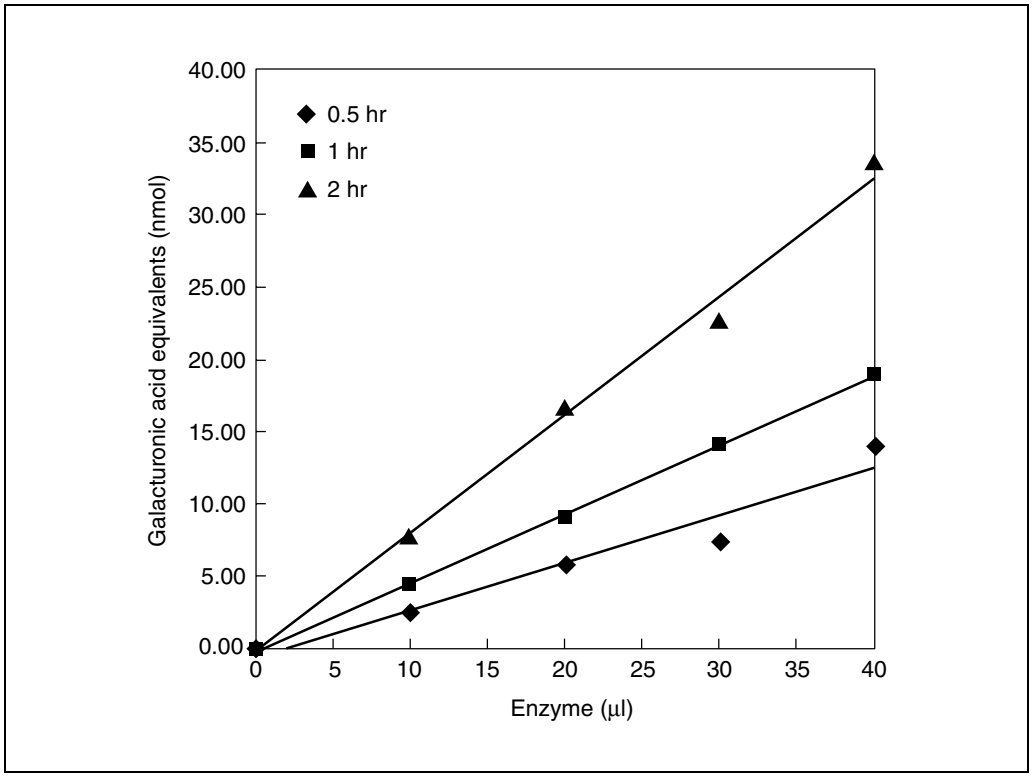

Figure C1.2.4 Relationship between enzyme load and amount of product generated (new reducing ends) at different extents of reaction.

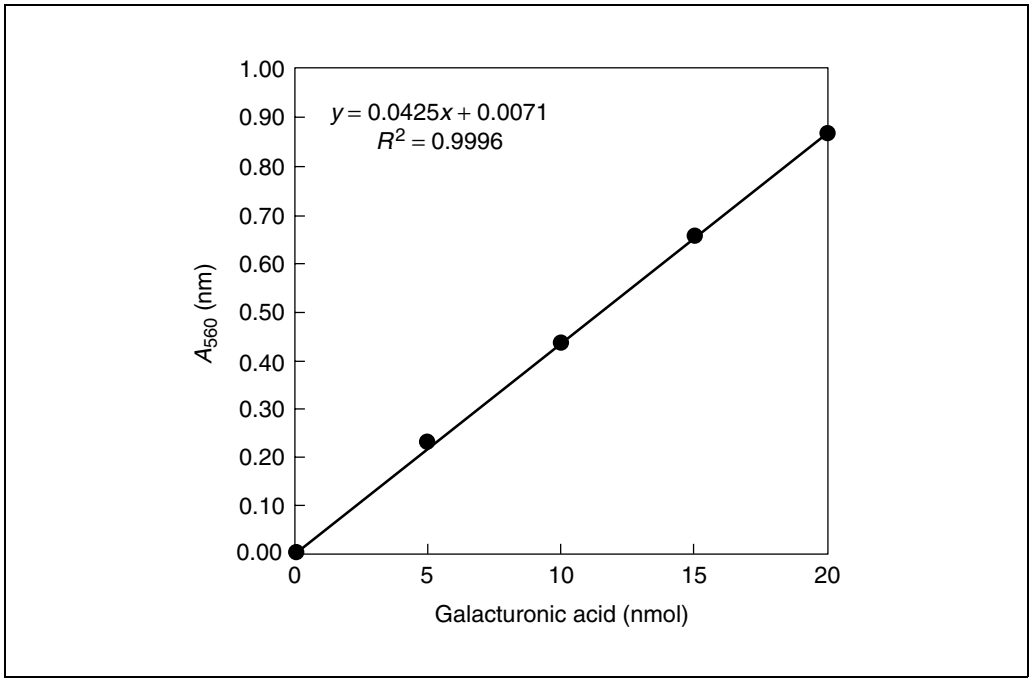

Figure C1.2.5 Calibration curve for the 2,2′-bicinchoninic acid reducing sugar–assay with galacturonic acid as the calibration standard and A_{560} as the analytical signal.

analysis of up to ~20 nmol reducing ends. If an enzyme assay generates product extending beyond this range, then the reaction mixture should be appropriately diluted prior to quantification of reducing sugars.

Time Considerations

A typical reducing sugar–based assay (Basic Protocol 1) takes ~4 hr following reagent and enzyme preparation. The assay is designed for use with enzyme preparations having relatively low activities. Enzyme preparations with higher activities could shorten the enzyme/substrate incubation period, the limitation being the need to generate sufficient reducing sugars for detection. The time required for the viscosity-based assay (Basic Protocol 2) is again somewhat dependent on the activity of the enzyme preparation being tested. As presented, the assay takes ~1 hr following enzyme and substrate preparation. To prepare an enzyme sample from tomatoes (Support Protocol), ~5 hr is needed.

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Activity Measurements of Proteinases Using Synthetic Substrates

BASIC PROTOCOL

A wide variety of synthetic substrates are now available for rapid and reliable in vitro assaying of activity of different proteases (peptidases), or for characterization of their substrate specificities. They are made up of either single amino acids or short peptides in which the α-NH_2 and/or the COOH groups are substituted with a blocking group, usually through either amide or ester linkages. Both groups are blocked in substrates for proteinases (endopeptidases), but the acyl group is usually conjugated to a chromogenic or fluorogenic group. On bond cleavage, these groups are liberated and are conveniently monitored by the associated changes in absorbance or fluorescence. In substrates for exopeptidases, either the NH_2 group (carboxypeptidases) or the COOH group (aminopeptidases) is substituted with such blocking groups. The most frequently used NH_2- and COOH-group substituents are listed in Table C2.1.1. The suitability of a substrate for a given peptidase is determined by how well the amino acid sequence of the peptide moiety conforms with the substrate-binding specificity of the enzyme.

This unit focuses on assays for proteinases only, and specifically on those that use paranitroanilide (p-NA) acyl derivatives as substrates. Assays using p-NA as a chromogenic leaving group are the most common and convenient spectroscopic method for activity measurements for a large array of peptidases. Several such substrates are available for different serine and cysteine proteinases with different specificities, as well as some aminopeptidases. The protocol described below is used to assay chymotrypsin-like serine proteinases, using the substrate Suc-AlaAlaProPhe-p-NA, which is also a good substrate for determining activity of several other proteinases, such as subtilisins, cathepsin G, and elastase II. The procedure also applies to assays using other p-NA substrates for different proteases (Table C2.1.2), although adjustments of pH, buffer composition, and substrate concentrations are required in accordance with the kinetic characteristics of the enzyme being studied.

In this assay, enzymes with chymotrypsin-like specificity readily bind the side chain of Phe at the primary substrate binding site (S1) and subsequently hydrolyze the adjacent amide bond linking the Phe residue to the p-NA moiety. On cleavage, the release of p-NA is measured by the increase in absorbance at 410 nm (ε_{410} = 8480 M^{-1} cm^{-1}) with time, using a recording spectrophotometer.

Table C2.1.1 Common Amino and Carboxyl Substituents Used with Various Synthetic Amino Acid and Peptide Substrates for Peptidases

Amino-group substituents	Carboxyl-group substituents
o-Aminobenzoyl (Abz)	7-Amido-4-methylcoumarin (AMC)
Acetyl (Ac)	7-Amino-trifluoromethylcoumarin (AFC)
Benzoyl (Bz)	Ethyl ester (OEt)
Benzyloxycarbonyl (Z)	Methyl ester (OMe)
t-Butyloxycarbonyl (Boc)	2-Naphtylamide (2NA)
Furylacryloyl (Fa)	*p*-Nitroanilide (p-NA)
Methoxysuccinyl (MeOSuc)	*p*-Nitrophenyl ester (ONp)
Pyroglutamate (Pyr)	Thiobenzyl ester (SBzl)
Succinyl (Suc)	

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Table C2.1.2 Examples of Synthetic Substrates[a] for Selected Proteinases

Enzyme	Type	Substrates
Brachyurin	Serine	Suc-AlaAlaProPhe-p-NA
		Suc-AlaAlaProArg-p-NA
		Suc-AlaAlaProPhe-SBzl
		Suc-AlaAlaProArg-SBzl
Bromelain	Cysteine	Z-ArgArg-AMC
Cathepsin B	Cysteine	Z-Arg-p-NA
		Z-ArgArg-p-NA
		Z-PheArg-p-NA
		Z-ArgArg-AMC
		Z-PheArg-AMC
Cathepsin G	Serine	Suc-AlaAlaProPhe-p-NA
		Suc-AlaAlaProPhe-SBzl
		Suc-PheLeuPhe-p-NA
		Suc-PheLeuPhe-SBz
		MeOSuc-AlaAlaProMet-p-NA
Cathepsin H	Cysteine	Bz-Arg-p-NA
		Bz-Arg-AMC
		H-Arg-p-NA
		H-Arg-AMC
Cathepsin L	Cysteine	Z-PheArg-p-NA
		Z-PheArg-AMC
Chymopapain	Cysteine	Bz-Arg-p-NA
		Z-PheArg-AMC
Chymotrypsin	Serine	Suc-Phe-p-NA
		Suc-GlyGlyPhe-p-NA
		Suc-AlaAlaProPhe-p-NA
		Suc-AlaAlaProPhe-SBzl
		Suc-AlaAlaProPhe-AMC
Elastases	Serine	Suc-AlaAlaAla-p-NA
		Suc-AlaAlaAla-AMC
		Ac-AlaProAla-p-NA
		Ac-AlaProAla-AMC
		Suc-AlaAlaVal-p-NA
		Suc-AlaAlaProPhe-p-NA
		Suc-AlaAlaProPhe-AMC
		Suc-AlaAlaProLeu-p-NA
		MeOSuc-AlaAlaProVal-p-NA
		MeOSuc-AlaAlaProVal-AMC
		Suc-AlaTyrLeuVal-p-NA
Ficain	Cysteine	Bz-Arg-p-NA
		Z-PheArg-p-NA
		Z-PheArg-AMC
		Bz-PheValArg-p-NA
		Bz-PheValArg-AMC
Papain	Cysteine	Bz-PheValArg
		Bz-PheValArg-AMC
		Z-PheArg-p-NA
		Z-PheArg-AMC

continued

Table C2.1.2 Examples of Synthetic Substrates[a] for Selected Proteinases, continued

Enzyme	Type	Substrates
Proteinase K	Serine	Suc-AlaAlaAla-p-NA
		Suc-AlaAlaAla-AMC
		Suc-AlaAlaPhe-p-NA
		Suc-AlaAlaProPhe-p-NA
Subtilisins	Serine	Suc-AlaAlaProPhe-p-NA
		Suc-AlaAlaProPhe-SBzl
		Suc-AlaAlaProPhe-AMC
		Suc-AlaAlaPhe-p-NA
		Z-GlyGlyLeu-p-NA
Thermolysin	Metallo	Suc-AlaAlaPhe-AMC
Trypsin	Serine	Bz-Arg-p-NA
		Bz-Arg-AMC
		Z-Arg-SBzl
		Z-GlyProArg-p-NA

[a]Major suppliers of synthetic proteinase substrates are: Bachem, Enzyme Systems Products, and Sigma.

The methods described are useful for assaying both purified proteinases, as well as for measurement of specific proteinase activities in raw aqueous extracts of different food or other biological materials. However, as these assays use spectroscopic detection techniques, it is highly important that such extracts be fully clarified before measurement.

Materials

Assay buffer (see recipe)
12.5 mM substrate solution (see recipe)
0.1 to 5 µM enzyme test solution
Buffer used for enzyme test solution: e.g., 50 to 100 mM Tris·Cl, pH 7.8 to 8.6 containing 10 mM $CaCl_2$ (see recipe for assay buffer)
Recording spectrophotometer equipped with thermostatted cuvette holder and connected to a chart recorder or appropriate data acquisition system for continuous recording of change in absorbance with time ($\Delta A/\Delta t$)
1-ml glass or quartz cuvettes

1. Turn on a recording spectrophotometer and set wavelength to 410 nm.
2. Turn on the circulating water bath and set to 25°C. Allow the instrument to stand ~15 min at this temperature before taking measurements.
3. To zero the spectrophotometer, successively pipet 950 µl assay buffer, 40 µl of 12.5 mM substrate solution, and 10 µl of the buffer in which the enzyme is dissolved into reference and sample cuvettes (total 1 ml each cuvette). Place in the cuvette holders and zero the instrument.

   Make sure that cuvette surfaces are clean.

4. Discard the contents of the sample cuvette and leave the reference cuvette in place.
5. Add 950 µl assay buffer and 40 µl of 12.5 mM substrate solution to the sample cuvette and place in the spectrophotometer. Leave 5 min at the set temperature.

6. Add 10 µl of 0.1 to 5 µM enzyme test solution and mix well with a small plastic spatula or by placing a small piece of Parafilm over the cuvette and inverting several times.

   The optimal concentration of the enzyme test solution depends on the proteinase being assayed, and must be determined experimentally. For chymotrypsin, 2.5 to 25 µg/ml (0.1 to 1 µM) is appropriate. Concentrations of 5 to 50 µg/ml are appropriate for most proteinases assayed with this procedure.

   When the level of enzyme activity or concentration is low, it may be necessary to change the composition of the assay mixture to increase the amount of enzyme test solution added (e.g., using 910 µl assay buffer, 40 µl substrate, and 50 µl enzyme test solution).

7. Immediately start recording absorbance at 410 nm, and continue for sufficient time to obtain a good linear tracing of the hydrolytic reaction.

   If the rise in absorbance is so rapid that it obscures reliable observation of linearity and of calculation of $\Delta A/\Delta t$, make appropriate dilutions of the enzyme solution and rerun the assay.

   For determination of activity from $\Delta A/\Delta t$, see Anticipated Results.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Assay buffer

12.11 g Tris·Cl (100 mM)
1.47 g $CaCl_2 \cdot 2H_2O$ (10 mM)
~900 ml water
Adjust to pH 7.8 to 8.6, as desired, with 3 M HCl
Dilute to 1000 ml with water
Store up to 2 months at 4°C

Substrate solution, 12.5 mM

Dissolve 78.1 mg Suc-AlaAlaProPhe-*p*-nitroanilide (Bachem, Enzyme Systems Products, or Sigma) in 10 ml dimethyl sulfoxide or dimethylformamide (12.5 mM). Store up to several months at 4°C, but check for the appearance of yellow color before use.

Yellow color indicates autolysis.

Using this stock solution, the final concentration of Suc-AlaAlaProPhe-p-NA in the assay mixture is 0.5 mM when diluted with buffer. Substrate solutions should be made fresh daily.

COMMENTARY

Background Information

Degradation of proteins by proteases has important implications for the quality of many foods. These include beneficial effects in the development of desirable attributes, such as texture and flavor, e.g. in cheese manufacturing, meat tenderization, beer brewing, soy and fish sauce manufacturing, and production of protein hydrolysates of various origins for different functional or nutritional purposes. The proteases involved may be endogenous to the foods or they may be added for specific purposes as isolated enzyme preparations. Proteases may also be secreted by microorganisms present in the foods, which may be beneficial, as in cheese maturation, or may have detrimental effects that lead to food spoilage. The degree of proteolysis in a food can be a decisive factor for its quality. It is therefore important for the food scientist to have ways to measure the activity of proteases in different food materials. Assays have been developed using either whole proteins or different synthetic substrates for measuring activity of various proteases.

The use of synthetic substrates to measure protease activity has some advantages over using whole proteins as substrates. One reason is that each peptide bond in a protein is in fact a potential substrate for different proteases (Sarath et al., 1989). Furthermore, as proteolysis proceeds, these substrates and their accessibility continuously change as the protein is broken down to smaller peptides, and hence the susceptibility of the peptide bonds to be cleaved changes. Different proteases hydrolyze peptide bonds at different rates depending on their substrate-binding specificities, which are determined by the exact topology of amino acid residues making up the specific binding sites in their three-dimensional structures. Factors such as pH affect the charge properties of the protein substrate as well as the enzyme, and may therefore critically influence substrate binding. They may do so to varying degrees for different proteases, which differ with respect to charge properties (e.g., isoelectric points). Comparisons of enzymatic properties of different proteases obtained with assays using whole-protein substrates may therefore be complicated by these factors.

Synthetic amino acid or peptide substrates, on the other hand, are designed so that only a specific bond is cleaved and the progress of the hydrolytic reaction can be monitored continuously, usually by spectroscopic means. Such substrates are synthesized based on known specificities of given proteases in order to design sensitive assays for the enzymes (Del Mar et al., 1979, 1980; Knight, 1995; Powers and Kam, 1995). Alternatively, a range of peptide substrates with specific sequences can be used to characterize substrate specificities of different proteases (Hedstrøm et al., 1994; Rheinnecker et al., 1994; Tsu and Craik, 1996). A broad range of synthetic amino acid and peptide substrates are now available from different suppliers for those experimental purposes. Table C2.1.2 lists examples of substrates used for selected proteinases. Previously, synthetic substrates of proteinases were usually made up of a single amino acid that was recognized at the primary binding site (S1) of the enzyme, with a blocking group attached to the α-NH_2 group, and with the acyl group conjugated to a leaving group through an ester or an amide bond that was cleaved by the enzyme. Most substrates are now synthesized as short peptide sequences on the N-terminal side of the bond cleaved. The peptide sequence confers selectivity to a substrate for a given proteinase, depending on how effectively the designed peptide sequence binds to the substrate-binding site of the enzyme. N-acylated peptide derivatives of p-nitroanilide (p-NA) are the most widely used synthetic chromogenic substrates for serine and cysteine proteinases (Table C2.1.2). Assays using p-NA substrates are sensitive and simple to perform (see Basic Protocol). The progress of the enzyme reaction is monitored continuously by measuring the increase in absorbance at 410 nm by p-nitroaniline (ε_{410} = 8480 $M^{-1}cm^{-1}$, Del Mar et al., 1979, or 8800 M^{-1} cm^{-1}, Erlanger et al., 1961) liberated on hydrolysis of the amide bond. A variety of peptide-pNA substrates are in use for assaying different proteases or for investigating their substrate specificities (Table C2.1.2).

Another group of synthetic substrates consists of peptide thioesters, which are used in assays for both serine proteinases and metalloproteinases (Powers and Kam, 1995). As with p-NA substrates, the peptide sequence determines the specificity of the substrate for the proteinase. However, because the catalytic efficiency (k_{cat}/K_m) of proteinases for cleaving thioester bonds is generally higher than that for amides, these substrates are usually more sensitive than the former. The thiol leaving group (thiobenzyl) that is formed on hydrolysis of the thioester bond is detected at low concentrations using either of the thiol reagents present in the assay mixture: 4,4′-dithiodipyridone or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). With 4,4′-dithiopyridone, the enzyme reaction rates are determined by measuring the increase in absorbance at 324 nm due to the formation of 4-thiopyridone (ε_{324} = 19,800 M^{-1} cm^{-1}). Absorbance is measured at 405 nm for 2-nitro-5-thiobenzoate (ε_{405} = 13,260 M^{-1} cm^{-1}) when DTNB is used to monitor the reaction (Powers and Kam, 1995). One advantage of using peptide thioester substrates for proteinase assays is their high sensitivity, which makes them a good alternative to p-NA substrates when detecting enzymes at very low concentrations. The selection of peptide thioesters that are commercially available is lower, however, than for p-NA substates.

Highly sensitive fluorogenic synthetic substrates are also available for a variety of proteinases. Those that are in most widespread use are short peptidyl derivatives in which the acyl group is conjugated to 7-amido-4-methylcoumarin (AMC). On cleavage of the amide bond by a proteinase, 7-amino-4-methylcoumarin is released, and the rate of the reaction can be monitored by its intense fluorescence, using a spectrofluorometer with an excitation wave-

length of 370 nm and emission wavelength of 460 nm (Knight, 1995). A large variety of AMC-substrates are commercially available. Because fluorescence methods are inherently sensitive, using these substrates could be the best choice when detecting low levels of given proteinases. Peptidyl 2-napthylamides have also been used as fluorogenic substrates for proteinases, but concerns about the carcinogenicity of the fluorophore, 2-napthylamine, limit the use of these substrates for routine proteinase assays.

Critical Parameters

Although the protocol described above is designed to assay serine proteinases having chymotrypsin-like activity, a selection of p-NA substrates (Table C2.1.1) are available for a range of different proteinases that can be assayed under a similar set of conditions. However, the assay conditions may have to be adjusted with respect to buffers, solutes, pH, substrate concentration, and assay temperature to best fit the properties of the proteinases being assayed. For instance, with Suc-AlaAlaProPhe-p-NA as a substrate, chymotrypsin is typically assayed at pH 7.8 (Del Mar et al., 1979), whereas subtilisins, which have more alkaline pH optima for activity, are typically assayed at pH 8.6 (Rheinnecker et al., 1994). The different enzymes may also differ with respect to kinetic parameters (K_m and k_{cat}) which will affect the choice of substrate concentrations used in an assay for a given enzyme. For Suc-AlaAlaProPhe-*p*-NA the K_m value for chymotrypsin is 43 mM (Del Mar et al., 1979), it is 150 mM for subtilisin BPN′ (Rheinnecker et al, 1994), but 2500 mM for human pancreatic elastase II (Del Mar et al, 1980) under standard assay conditions for the enzymes at 25°C. Thus, when using a substrate concentration of 0.5 mM in the assay for these enzymes, the observed velocities would be ~ 92%, ~77%, and ~17% of the maximal velocities, for chymotrypsin, subtilisin, BPN′, and elastase II, respectively. Thus the sensitivity of the assay for different enzymes varies as a result of differences in these parameters. It is therefore advisable to determine an appropriate substrate concentration for a given enzyme, by measuring the rates of enzyme reaction in a selected concentration range, such as 0.25 to 2.5 mM for Suc-AlaAlaProPhe-*p*-NA, before a final concentration of substrate is selected for an assay procedure. Also as assays are often not carried out at substrate concentrations corresponding to maximal velocities for the proteinases, it is important to determine the concentration of substrate being used. This can be done by directly measuring the absorbance of the substrate solution at 315 nm after mixing with assay buffer (ε_{315} = 14,000 M^{-1} cm^{-1}), or by measuring at 410 nm (ε_{410} = 8480 M^{-1} cm^{-1}) after total enzymatic hydrolysis of the substrate.

Anticipated Results

The spectrophotometer measures and displays the increase in absorbance at 410 nm as a function of time ($\Delta A/\Delta t$). Whether the output from the instrument is in the form of a strip chart or is collected by a computer, the reaction velocities are usually expressed in terms of change in concentration per unit time, or converted to specified units of enzyme activity. The International Unit (U) for enzyme activity is defined as the amount of enzyme that transforms 1 μmol substrate to product in 1 min under specified assay conditions. The SI unit for activity is the katal, which is defined as the amount of enzyme that transforms 1 mol substrate per second under specified conditions. Thus 1 U = 16.7 nkatal. To convert slopes ($\Delta A/\Delta t$ values) to velocities (v), the following equation is used:

$$v = \frac{\Delta A/\Delta t \times \mathrm{Vol} \times 10^6\,\mu\mathrm{M/M}}{\varepsilon_{410} \times l}$$

$$v = \frac{\Delta A/\Delta t \times 0.001\ \mathrm{liter} \times 10^6\,\mu\mathrm{M/M}}{8480\ \mathrm{M^{-1}\ cm^{-1}} \times 1\ \mathrm{cm}}$$
$$= \Delta A/\Delta t \times 0.118\ \mu\mathrm{mol/\,min\,(or\ U)}$$

The velocity as calculated is independent of volume; thus, to calculate the enzyme activity in terms of U/ml, the velocity is divided by the volume (ml) of enzyme solution used in the assay (0.01 ml in the above protocol), or by mg enzyme present in the assay mixture to obtain specific activity (U/mg).

Time Considerations

After the suggested temperature equilibration time, <5 min are needed to carry out each assay.

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Different aspects of handling, characterization, inhibition, and use of proteolytic enzymes.

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Peptidase Activity Assays Using Protein Substrates

The International Union of Biochemistry and Molecular Biology recommends that the term "peptidase" be used synonymously with the term "peptide hydrolase" (IUBMB, 1992). Thus, in this unit the term peptidase is used in reference to any enzyme that catalyzes the hydrolysis of peptide bonds, without distinguishing between exo- and endopeptidase activities. Peptidases may be assayed using native or modified proteins, peptides, or synthetic substrates. In this unit, the focus is on assays based on the hydrolysis of common, commercially available, protein substrates. Thus, the assays are not intended to be selective for a given peptidase; they are designed to provide estimates of overall peptidase activity. Other units in this publication focus on synthetic or "model" substrates, which can be designed for the measurement of specific endo- and/or exopeptidase activities.

STRATEGIC PLANNING

The following protocols may be viewed as comprising three operations. The first is the enzymatic reaction itself, the second is the simultaneous termination of the reaction and separation of enzyme-generated products from other protein/peptide components, and the third is product quantification. Variables that must be defined in the first operation include pH, buffer composition, temperature, and protein substrate. Traditional substrates for these assays are the caseins and denatured hemoglobin, or conjugates thereof (Stauffer, 1989). These proteins are convenient substrates for monitoring peptidase activity because fairly reproducible preparations are commercially available at relatively low cost and, in general, they are readily susceptible to proteolysis by a wide range of peptidases. The protocols in this unit, regardless of the substrate employed, are dependent on monitoring the generation of short peptides resulting from peptidase activity. Separation, prior to peptide quantification, is in all cases based on the differential solubility of peptides and proteins in trichloroacetic acid (TCA) solutions. Lower-molecular-weight peptides are more soluble in 3% to 5% TCA than are higher-molecular-weight peptides and proteins (Yvon et al., 1989). Quantitative estimates of the TCA-soluble peptides resulting from peptidase activity can be obtained by several methods (for methods to measure soluble protein, see Chapter B1).

In this unit, Basic Protocol 1 presents a procedure using casein as substrate. The Alternate Protocol describes the modification of this procedure for use with a denatured hemoglobin substrate. Basic Protocol 2 presents a procedure using a chromaphore-conjugated casein derivative, azocasein. For quantitation, the authors have chosen to use either the BCA-based colorimetric assay (*UNIT B1.1*) for soluble protein/peptides (in Basic Protocol 1) or the intrinsic absorbance of the chromaphore-conjugated peptide products (in Basic Protocol 2).

The assays presented in this unit assume that the analyst is working with a soluble enzyme preparation. Enzyme preparations of this type are often made by rupturing cell walls and/or membranes of the target tissue followed by enzyme extraction with an appropriately buffered solution. The extraction buffer should maintain pH and ionic strength in a range that is compatible with the enzyme's stability. Tissue disruption and enzyme extraction are often done simultaneously by homogenizing the target tissue in the extraction buffer and then separating the solids fraction by either centrifugation or filtration. It is expected that all enzyme preparations will be kept cold to minimize activity loss. In some cases it may be beneficial to add stabilizing agents to the extraction buffer,

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like a metal chelator (such as 1 mM EDTA) or a reducing agent (such as 1 mM dithiothreitol). It is important to make sure that all components included in the extraction buffer are compatible with each phase of the assay. A sample protocol for enzyme preparation from kiwi fruit is presented in the Support Protocol.

BASIC PROTOCOL 1

DETERMINATION OF PEPTIDASE ACTIVITY USING A CASEIN SUBSTRATE

This traditional assay is based on the ability of peptidases to generate 5% trichloroacetic acid (TCA)–soluble peptides from casein. Casein preparations suitable for use as peptidase substrates are commercially available (e.g., Hammerstein grade, ICN Biomedicals). They are typically prepared by acid precipitation of bovine skim milk, the proteinaceous precipitate being casein. The different casein preparations are purified, to a greater or lesser extent, by cycles of dissolving in alkali and reprecipitating. Acidulants commonly used for these processes include lactic, hydrochloric, and sulfuric acids (Walstra et al., 1999); the original "Hammerstein casein" method used acetic acid as the acidulant (McMeekin, 1954). Casein preparations typically consist of four principal gene products, α_{s1}, α_{s2}, β, and κ, in relative amounts of 40:10:35:12, respectively (Mulvihill, 1994).

Enzyme activities are based on rates of casein hydrolysis under defined conditions. The products of casein hydrolysis, as defined in this protocol, are those peptides soluble in 5% TCA that can be detected by the bicinchoninic acid (BCA) protein assay (*UNIT B1.1*). The amount of TCA-soluble peptide generated during the course of the reaction can actually be quantified by any one of several protein/peptide assays. The color yield in these assays is assumed to be proportional to the amount of peptide in solution. The amount of product/peptide in the reaction mixture is often reported as bovine serum albumin (BSA) equivalents—since standard curves based on this protein may be used to calibrate the assay. Thus, activity units can be expressed as the amount of BSA equivalents generated per unit time.

Materials

Enzyme preparation (see Strategic Planning and Support Protocol)
1% (w/v) casein substrate solution (see recipe)
10% (w/v) trichloroacetic acid (TCA)

30°C water bath
10-ml glass test tubes

Additional reagents and equipment for the bicinchoninic acid (BCA) protein assay (*UNIT B1.1*)

Perform enzymatic reaction

1. Bring 1% casein substrate solution to reaction temperature (30°C).
2. Set up a series of eighteen 10-ml glass test tubes for each enzyme preparation to be analyzed (i.e., enough for triplicate sample tubes and triplicate enzyme-substrate blank tubes for each of the three enzyme concentrations tested).
3. Add 0.1 ml of appropriate enzyme preparation to each of the eighteen test tubes. Place the nine sample tubes in the 30°C water bath for temperature equilibration (~5 min). Leave the nine enzyme-substrate blank tubes on ice.
4. Once equilibrated, initiate reactions in just the nine sample tubes by adding 0.9 ml of 1% casein substrate solution, with gentle mixing. Incubate 10 min at 30°C. After the sample tubes have incubated 5 min, place the enzyme-substrate blank tubes in the 30°C water bath to equilibrate.

Terminate reaction and separate components

5. At the completion of the 10-min reaction period, terminate the reactions by adding 1 ml of 10% TCA to each of the eighteen (sample and blank) tubes.

   This will inactivate the enzyme and thus terminate the reaction. TCA also terminates potential autolysis, which may occur in the blanks.

6. Immediately after TCA addition, add 0.9 ml casein substrate solution to each of the nine enzyme-substrate blank tubes.

   Peptidase activity is prevented in the blanks by inactivating the enzyme prior to adding substrate.

7. Remove all tubes from water bath and allow to stand 20 min at room temperature.
8. Pellet the precipitated material by centrifuging 5 min at $8000 \times g$, room temperature.

Quantify products

9. Remove sufficient supernatant to allow measurement of the soluble peptide by the BCA protein assay (*UNIT B1.1*), using bovine serum albumin (BSA; 20 to 120 µg/assay) as a standard.

   Many methods are available for measuring TCA-soluble peptides. Possibly the easiest is to measure the absorbance of the solution at 280 nm, as the absorbance at this wavelength is a function of the aromatic amino acid content of the solution. This approach requires a UV spectrophotometer, and the sensitivity of the assay is likely to be lower than that of some of the colorimetric assays. There are also several colorimetric peptide assays that can be applied to this type of peptidase assay, such as the Biuret, Lowry, and Bradford dye-binding methods (for comparison see Piyachomkwan and Penner, 1995). All of these methods measure a relative value rather than an absolute amount of peptide in solution. The results should thus be reported in terms of "equivalents," such as BSA equivalents when using a calibration curve prepared using a BSA standard solution.

10. Subtract appropriate mean blank values from mean sample values of the same enzyme concentration to obtain the average amount of product peptide produced during the 10-min reaction period.
11. Report activity as BSA equivalents generated per unit time.

    A "unit" could thus be defined as the amount of enzyme that produces 1 µg BSA equivalents per minute, under the conditions defined for a particular assay.

DETERMINATION OF PEPTIDASE ACTIVITY USING A HEMOGLOBIN SUBSTRATE

ALTERNATE PROTOCOL

Hemoglobin is also widely used as a substrate for monitoring general peptidase activity. It is particularly useful for measuring activities at pH <6, since casein is insoluble in that pH region. The hemoglobin used for such assays is almost always referred to as "denatured." Denatured hemoglobin is used because the native form of the protein is relatively resistant to peptidase activity. Denatured hemoglobin can be prepared in several ways; the different methods of preparation will not necessarily result in substrates that are equally susceptible to proteolytic activity (Schlamowitz and Peterson, 1959). The most common of these substrates is urea-denatured hemoglobin (Anson, 1938). It is typically prepared under alkaline conditions (see Reagents and Solutions; adapted from Worthington, 1993). Assays using this substrate are, however, complicated by the presence of urea in the reaction mixture, which can lead to denaturation of the peptidase itself. Thus, assays using urea-denatured hemoglobin should be completed in as short a time as possible to minimize the amount of time the enzyme is exposed to urea. It has been suggested that assays not exceed 10 min (Sarath et al., 2001).

BASIC PROTOCOL 2

DETERMINATION OF PEPTIDASE ACTIVITY USING AN AZOCASEIN SUBSTRATE

Reporter group–labeled proteins are often effective substrates for detecting peptidase activity (Sarath et al., 2001). Azoproteins, which are chromophore-labeled proteins, have been used for this purpose for many years (Charney and Tomarelli, 1947). The term azoprotein, when used in relation to generic peptidase substrates, most commonly refers to the sulfanilic-acid-azo or the sulfanilamide-azo derivative of the protein. They are prepared by coupling the corresponding diazo compound with the native protein under alkaline conditions (for examples, see Hazen et al., 1965, or Jones et al., 1998). The resulting modified protein is expected to have azo-derivatized tyrosine and histidine residues; other amino acids are also susceptible to modification (Means and Feeney, 1971). If diazobenzenesulfonic acid, prepared by diazotization of sulfanilic acid, couples with the phenolic moieties of a protein's tyrosyl residues, then the resulting moieties will be substituted by azophenylsulfonate groups (a bis-coupled phenolic group is shown in Fig. C2.2.1; Haurowitz, 1963).

Azoproteins are particularly useful for detecting peptidase activity due to their rather intense color (in the orange range). Azocasein is one of the most widely used azoproteins for this purpose. It is commercially available (e.g., Sigma) and easy to use. The major advantage of using the azo-modified protein is that the peptide-quantifying (color-generating) reaction, following TCA precipitation in the traditional assay, is not necessary. Instead, the absorbance inherent in the TCA-soluble chromophore-labeled peptides is the basis of quantification. Thus, a simple visible-wavelength spectrophotometer (colorimeter) can be used to monitor the extent of proteolysis without the need for a color-generating reaction (although the pH of the TCA-containing soluble phase is typically raised to increase the assay's sensitivity at 440 nm; Planter, 1991).

Materials

Enzyme preparation (see Strategic Planning and Support Protocol)
1.5% (w/v) azocasein substrate solution (see recipe)
10% (w/v) trichloroacetic acid
Color generating solution: 1 M NaOH

30°C water bath
5- and 10-ml glass test tubes
Spectrophotometer with 1-cm glass or plastic cuvettes

Perform enzymatic reaction

1. Bring 1.5% azocasein substrate solution to reaction temperature (30°C).
2. Set up a series of eighteen 10-ml glass test tubes for each enzyme preparation to be analyzed (enough for triplicate sample tubes and triplicate enzyme-substrate blank tubes for each of the three enzyme concentrations tested).

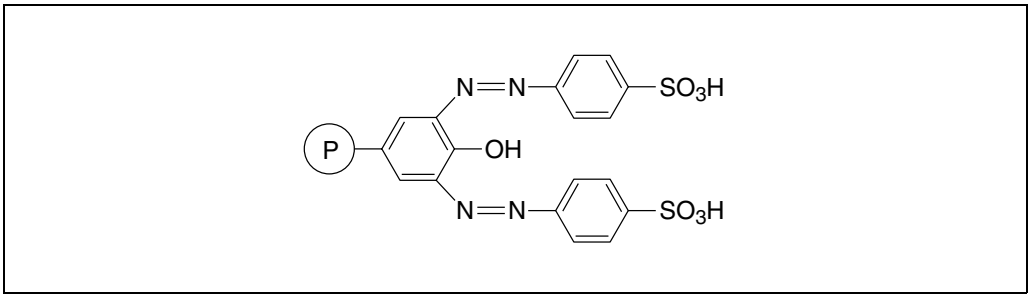

Figure C2.2.1 Azophenylsulfonate substitution of protein tyrosyl side chain.

3. Add 0.2 ml of appropriate enzyme preparation to each of the eighteen test tubes. Place the nine sample tubes in the 30°C water bath for temperature equilibration (~5 min). Leave the nine enzyme-substrate blank tubes on ice.

4. Once equilibrated, initiate reactions in just the nine sample tubes by adding 0.80 ml of 1% azocasein substrate solution and mixing gently. Incubate 10 min at 30°C. After the sample tubes have incubated 5 min, place the enzyme-substrate blank tubes in the 30°C water bath to equilibrate.

Terminate reaction and separate components

5. At the completion of the 10-min reaction period, terminate the reactions by adding 1 ml of 10% TCA to each of the eighteen (sample and blank) tubes.

   This will inactivate the enzyme and thus terminate the reaction. TCA addition also terminates potential autolysis, which may occur in the blanks.

6. Immediately after TCA addition, add 0.8 ml of azocasein substrate solution to each of the nine enzyme-substrate blank tubes.

   Peptidase activity is prevented in the blanks by inactivating the enzyme prior to adding substrate.

7. Remove all tubes from water bath and allow to stand 20 min at room temperature.

8. Pellet the precipitated material by centrifuging 5 min at 8000 × *g*, room temperature.

Quantify products

9. Transfer 1 ml of each resulting supernatant to a 5-ml glass test tube containing 1 ml of 1.0 N NaOH.

10. Measure absorbance at 440 nm against water as a reference.

11. Subtracting the appropriate mean blank value from the mean sample value for each of the enzyme concentrations tested to give the amount of peptide product produced during the 10-min reaction period.

12. Determine the activity units, where a unit is defined as that amount of enzyme required to produce an absorbance change of 1.0 per min in a 1-cm cuvette.

ENZYME PREPARATION EXAMPLE: KIWI FRUIT

SUPPORT PROTOCOL

In this protocol, enzyme is prepared from a single kiwi fruit, the fruit of the Chinese gooseberry (*Actinidia chinensis*), purchased at a local grocery store. The predominant peptidase of the kiwi fruit is a cysteine peptidase called actinidain.

Materials

Kiwi fruit
0.1 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*)

Kitchen spoon
Freezer storage bags
Cheesecloth

1. Cut fruit in half and scoop out the flesh using a kitchen spoon.

2. Place tissue in a preweighed freezer storage bag, seal, and then weigh to determine the weight of the flesh (by difference).

3. Pulp the flesh by hand squeezing and place the sealed bag in a freezer until ready for use (typically overnight).
4. Allow pulp to thaw at room temperature for ~30 min.
5. Add an equal weight of 0.1 M sodium phosphate buffer, pH 7.0, to the pulp and homogenize the mixture by hand (~5 min).
6. Filter the resulting suspension through cheesecloth.
7. Centrifuge the cloudy filtrate 20 min at 10,000 × *g*, 4°C, and collect the supernatant as the enzyme preparation.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Azocasein substrate solution, 1.5% (w/v)

Dissolve 1.5 g azocasein (Sigma) in 0.05 M sodium phosphate buffer, pH 7 (*APPENDIX 2A*) by stirring and, if necessary, gentle heating (to ~50°C). Bring the final volume to 100 ml with the same buffer. Store up to 5 days at 4°C.

Casein substrate solution, 1% (w/v)

Dissolve 1 g casein (Hammerstein grade, ICN Biomedicals) in 50 ml of 0.01 N NaOH by stirring and, if necessary, gentle heating (to ~50°C). With continued stirring, add 40 ml of 0.25 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*) and bring to 100 ml with deionized water. Store up to 5 days at 4°C.

The pH of this solution will be ~7.1. If a different pH is desired, then adjust the pH to the desired value prior to bringing the final volume to 100 ml. The pH of the reaction mixture should be kept above 6 to prevent precipitation of casein.

Preparing a 1% casein solution is somewhat tricky because dried casein preparations are typically only sparingly soluble in neutral aqueous systems. This method, which uses alkaline conditions to prepare a casein sol, should work for most purposes. The casein substrate solutions used for peptidase assays are often more accurately described as substrate suspensions, since these preparations are typically not true solutions—the casein component being of a colloidal nature.

Denatured hemoglobin, 2% (w/v)

Dissolve 2.0 g bovine hemoglobin (Sigma) in 35 ml reagent-grade water. Add 36.0 g urea and 16 ml of 0.5 M NaOH. Stir 30 min at room temperature. Add buffer (typically sodium phosphate; see *APPENDIX 2A*), adjust pH to that appropriate for the enzyme system under investigation, and then bring to 100 ml with water. Store up to 5 days at 4°C.

COMMENTARY

Background Information

Peptidases have been referred to as "the most important group of enzymes in the food processing industry" (Whitaker, 1994). Their integral role is in catalyzing the depolymerization of proteins, which are themselves significant contributors to the functional properties of foods. Endogenous and supplemental peptidases are both important; the former affecting such properties as gel strength, foam and emulsion stability, and flavor profiles; the latter being used in applications such as meat tenderization and the initiation of curd formation in cheese manufacture.

All peptidases catalyze the general reaction depicted in Figure C2.2.2, the hydrolysis of a peptide bond. The different peptidases are unique with respect to their specificity; that is, their ability to accommodate particular sets of amino acids in the vicinity of a potentially scissile peptide bond. Some peptidases have very broad specificities, such as papain, which has few limi-

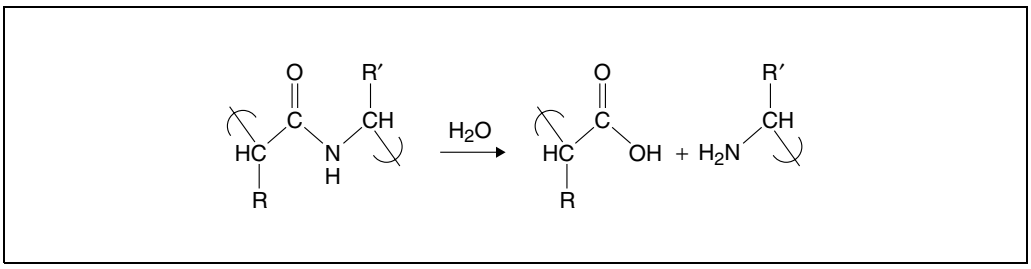

Figure C2.2.2 Peptidase reaction: hydrolysis of peptide bond.

tations on the amino acids in the vicinity of the scissile bond (Keil, 1992; Rawlings and Barrett, 1994). Others are highly specific, such as renin, which is specific for a particular locus in the protein angiotensin, its natural substrate (Slater, 1981; Keil, 1992).

Peptidases are often classified as either exopeptidases or endopeptidases, depending on the positional specificity of the bonds they hydrolyze. Exopeptidases act at peptide bonds located at either the N or C terminus of the protein. Those acting at the C terminus are referred to as carboxypeptidases, those acting at the N terminus as aminopeptidases. Endopeptidases, on the other hand, act at peptide bonds internal to the polypeptide chain.

Peptidases are further classified based on their catalytic mechanism. The following four mechanistic classes are well established (Dunn, 2001).

1. Serine peptidases: the catalytic apparatus includes a nucleophilic serine residue.

2. Cysteine peptidases: the catalytic apparatus includes a nucleophilic cysteine residue.

3. Metallopeptidases: the catalytic apparatus includes a metal ion.

4. Aspartic proteases: the catalytic apparatus includes two aspartic acid residues.

This nomenclature scheme, based on active site chemistry, is complimentary to the endo/exo nomenclature described above. Thus, the two schemes are frequently combined. For example, trypsin (a digestive enzyme) is often descriptively referred to as a serine endopeptidase.

The assays presented in this unit are far more sensitive for endopeptidases than for exopeptidases, because the assays are not designed to measure the actual number of catalytic events resulting from peptidase activity. Instead, the assays attempt to measure the amount of TCA-soluble peptide generated during a given reaction period, assuming that this value will be a function of the amount of active endopeptidase present in the reaction mixture. These TCA-soluble peptides can range from 2 to >20 amino acids in length (Yvon et al., 1989). The quantification methods used to detect these peptides, such as the BCA peptide/protein assay, are not well suited for the measurement of free (albeit TCA-soluble) amino acids, which are (along with dipeptides) the expected products resulting from exopeptidase activity. Hence, exopeptidase activity may go largely undetected with this type of assay. The azosubstrates present yet another barrier for exopeptidase activity, because exopeptidases are not expected to process the azo-derivatized amino acids, and hence should generate little to no color. The endopeptidases apparently work by hydrolyzing peptide linkages on either side of the derivatized amino acid, thus generating a chromaphore-labeled peptide from the azosubstrate.

Critical Parameters

Quantitative measurements of enzyme activity are based on rate assays. Hence, all experimental parameters that may affect the rate of an enzyme-catalyzed reaction, including pH, ionic strength, buffer composition, and temperature, are to be defined. These parameters are typically chosen to coincide with optimum or physiologically/technologically relevant conditions. Furthermore, it is generally assumed that quantitative activity measurements reflect initial-velocity kinetics. This means that the analyst must establish, for all experimental permutations, that initial rates are indeed being measured. These topics, as well as other information related to the design of enzyme assays, are discussed in *UNIT C1.1*.

A unique aspect of using protein substrates is that the substrates themselves are so complex. Even a relatively small protein, having say 100 amino acids, has 99 potentially susceptible peptide bonds—and each of these bonds is expected to be unique. This hypothetical protein thus has 99 structurally unique substrates (potential sites for catalysis). The environment surrounding each of the protein's peptide bonds will be dependent on the tertiary structure of the protein, so one must consider, for example, native

versus denatured forms of the protein. Furthermore, initially unavailable (e.g., buried) peptide bonds may become accessible as a protein is depolymerized; at a minimum the physicochemical environment of the different peptide linkages will change as the protein is depolymerized. Thus, one can expect that the substrate profile will change over the course of the reaction. When considering the azoproteins, the substrate profile will also be a function of the extent of azo-coupling. Here the rational assumption is made that a derivatized amino acid, in the vicinity of a peptide bond, will perturb the natural protein-substrate interactions that occur at that peptide bond.

The peptide/protein quantification methods used in these assays are somewhat imprecise, because the color yield per mole or per milligram peptide is not consistent between peptides or proteins of different composition (Davis, 1988). Amounts of product are thus typically reported in terms of BSA equivalents, because calibration curves are typically prepared with this protein, and, again, absolute mole or absolute mass values are not readily obtained. This same limitation applies to the azosubstrates, since the color yield per mass peptide will be dependent on that given peptide's extent of derivitization.

The assays presented in this unit are best used for the detection of peptidase activity and for estimating the relative activity of test samples having the same peptidase composition. The authors do not recommend using these assays for quantitative comparisons of the molar amounts of different peptidases, because different peptidases, due to their unique specificities, may have vastly different activities on different substrates. It is not inconceivable for preparation "A," consisting of peptidase "A," to show higher activity than preparation "B," consisting of peptidase "B," when assayed with protein substrate "1" (say, casein), but lower activity than preparation "B" when assayed with protein substrate "2" (say, hemoglobin). This limitation suggests that comparative studies with different enzymes should include assays with different substrates, and, even then, be interpreted with caution.

Anticipated Results

Experimental conditions resulting in a linear or near-linear relationship between rate of product formation and enzyme concentration are advantageous. Such a relationship allows the analyst to back-calculate relative enzyme activities by simply accounting for dilution factors. Figure C2.2.3, using a commercial peptidase preparation, shows that such a linear relationship can be expected at lower enzyme concentrations.

A model experiment using a "crude" enzyme preparation from kiwi fruit (see Support Protocol) is presented in the hope that the approach may serve as a template for the design of other assays, and the results provide an example of what may be expected with raw biological materials, albeit the kiwi fruit is a raw product with relatively high peptidase activity (Lewis and Luh, 1988). Assay conditions and substrate preparation were performed as in Basic Protocol 1, with the pH of the substrate adjusted to 7.0. Enzyme preparation was performed as in the Support Protocol. The same

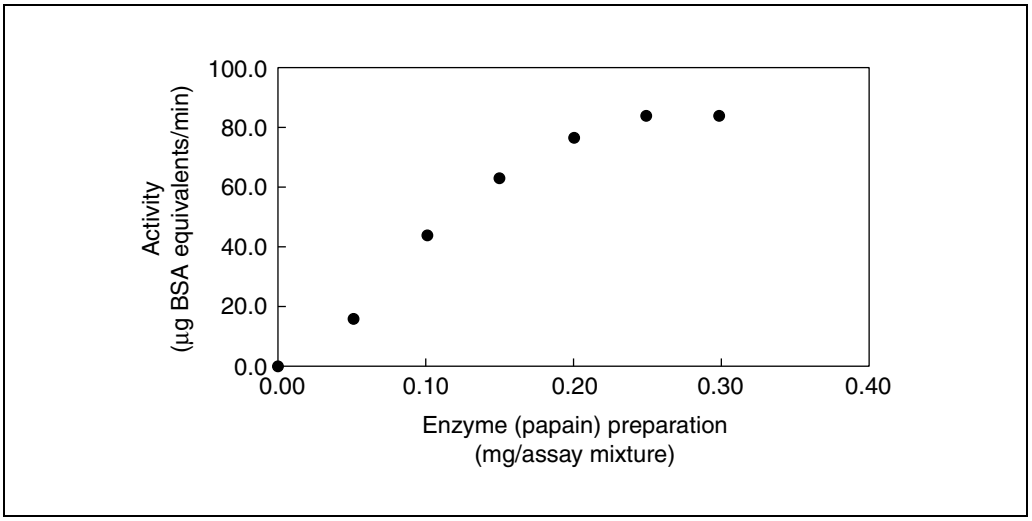

Figure C2.2.3 Relationship between amount of enzyme in reaction mixture (mg papain preparation from Sigma) and measured activity. Results were obtained using Basic Protocol 1 with Hammerstein casein substrate (ICN Biomedicals).

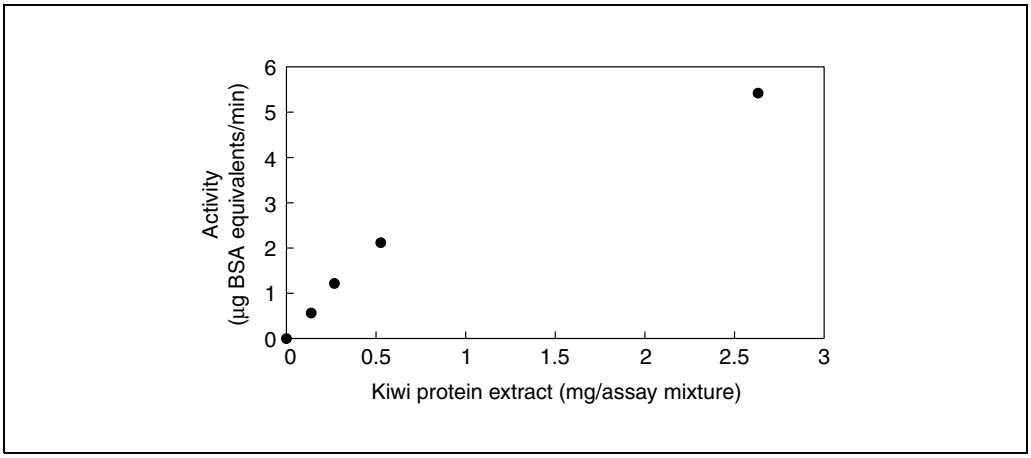

Figure C2.2.4 Enzyme extract activity profiles for raw kiwi fruit. Results were obtained using Basic Protocol 1 with Hammerstein casein substrate (ICN Biomedicals).

buffer (0.1 M NaH_2PO_4, pH 7.0) was used for both enzyme and substrate preparation. Figure C2.2.4 illustrates the relationship between measured peptidase activity and volume of kiwi fruit extract included in the reaction mixture. The relationship is essentially linear at the lower enzyme concentrations (lower amounts of extract added to the reaction mixture). The linear relationship begins to break down at higher enzyme concentrations. The data provide a first estimate of the range of kiwi fruit peptidase activities that are appropriate for analysis using Basic Protocol 1. Clearly, the analyst would want to work with enzyme levels that fall within the linear range of the assay.

The amount of active enzyme associated with a given enzyme preparation is typically reported as units of activity per milliliter, units of activity per milligram, or units of activity per milligram protein. The latter expression (units/mg protein) is referred to as the preparation's specific activity (Segel, 1975). Measured specific activities for the peptidase preparation employed in this experiment are given in Table C2.2.1. Notice that values calculated from data obtained in the linear range of the assay are in good agreement, as would be expected. In contrast, the value associated with the highest enzyme concentration tested is significantly below those obtained at the lower enzyme concentrations—again illustrating the importance of working in the linear portion of the assay.

Time Considerations

A typical assay with the casein or hemoglobin substrates (Basic Protocol 1 or Alternate Protocol) takes ~2 hr following reagent and enzyme preparation. Assays employing the azo-substrates (Basic Protocol 2) are considerably shorter, ~1 hr, since the color development step is not necessary.

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Table C2.2.1 Specific Activity Values for a Single Kiwi Fruit Peptidase Extract Measured at Different Enzyme/Protein Concentrations Using Basic Protocol 1

Enzyme extract (µl/reaction mixture)	Activity[a] (µg BSA eq/min)	Protein[b] (mg/reaction mixture)	Specific activity (µg BSA eq/min/mg protein)
0	0.0	0	0.0
5	0.5	0.32	4.2
10	1.2	0.263	4.6
20	2.1	0.527	4.0
100	5.4	2.63	2.0

[a]Measured as in Basic Protocol 1 with casein substrate.

[b]Measured by the BCA protein assay of Smith et al. (1985).

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This text is devoted solely to peptidase enzymes, although the information can be extrapolated to other systems. The text covers a wide range of topics, including assay methods, mechanisms of action, inhibition, and technological applications of peptidases, in a relatively straightforward manner.

Eisenthal, R. and Danson, M.J. (eds.) 1992. Enzyme Assays: A Practical Approach. Oxford University Press, Oxford.

This text covers the design and execution of enzyme assays. Chapters 1, 9, and 11 ("Principles of enzyme assay and kinetic studies," "Techniques of enzyme extraction," and "Buffers and the determination of protein concentration," respectively) are particularly relevant to this unit.

Whitaker, J.R. 1994. Principles of Enzymology for the Food Sciences, 2nd ed. Marcel Dekker, New York.

A classic text, directed at the food sciences, covering the fundamental principles of enzymology. Chapters covering enzyme purification, pH effects, temperature effects, enzyme inhibitors, and the proteolytic enzymes are particularly relevant to this unit.

Wong, D.W.S. 1995. Food Enzymes: Structure and Mechanism. Chapman and Hall, London.

This text is a good source of information on the chemical mechanisms underlying the different modes of peptidase catalysis. Three important enzymes are covered: subtilisin, a serine endopeptidase; papain, a cysteine endopeptidase; and chymosin, an aspartic endopeptidase.

Contributed by Ozlem Akpinar and Michael H. Penner
Oregon State University
Corvallis, Oregon

Lipase Assays

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) have broad applications in the food, oleochemical, pharmaceutical, and detergent industries, as well as in diagnostic settings (Hafkenscheid et al., 1983; Schmid and Verger, 1998). Over 100 lipases have been characterized to some extent, and more than 30 of these are commercially available. The identification of novel sources of lipases with unique patterns of reaction selectivity remains a strategic objective of lipase studies. Various levels of sophistication are required in the assessment of lipase activities, and they increase as one attains the differential goals of (1) screening for activity (qualitative and semi-quantitative); (2) quantifying activity; and (3) characterizing kinetic patterns of selectivity. The focus of this unit will be quantification of lipase activity, since methods developed for this purpose can often be adapted for the other two purposes as well.

Lipases are unusual hydrolytic enzymes because they act on substrates providing an interface (with few exceptions). This feature has been historically used to distinguish lipases from esterases, the latter of which act on substrates in true solution (Jensen, 1983). The distinction of lipases as interfacial catalysts can make kinetic characterization a challenge, because relevant substrate concentrations are expressed in terms of area and not concentration.

Even though esterases and lipases differ in their ability to act at interfaces, some assays can be used to measure both types of enzyme activities, since they share the general ability to hydrolyze carboxyl esters of various alcohols. In general, the use of water-soluble substrates (generally, shorter acyl chain length derivatives) is considered diagnostic for esterases, and the use of water-insoluble substrates (longer acyl chain length derivatives) is considered diagnostic for lipases. Of the three most commonly used assays, two exploit the ability to measure the free carboxylic acid or fatty acid residues liberated during lipase hydrolysis of native substrates, whether it be by titration of the released acid (Basic Protocol 1), or detection of the fatty acids by complexation with a colorimetric reagent of cupric acetate (Basic Protocol 2). Alternatively, the use of *p*-nitrophenyl acyl esters as chromogenic substrate analogs provides for a continuous, spectrophotometric assay (Basic Protocol 3). Throughout this unit, the term "fatty acid" will be used instead of the popular term "free fatty acid" since the former is preferred by IUPAC-IUB (1977), according to rules of nomenclature.

TITRIMETRIC DETERMINATION OF LIPASE ACTIVITY

BASIC PROTOCOL 1

In this procedure, native substrates (triacylglycerols) are hydrolyzed to yield fatty acids. Subsamples are withdrawn from reactive mixtures at predetermined intervals, and reactivity is quenched by the addition of ethanol. The amount of fatty acids released during the reaction is determined by direct titration with NaOH to a thymolphthalein end point.

Materials

95% (v/v) ethanol
1% (w/v) thymolphthalein indicator
Olive oil/gum arabic emulsion substrate (see recipe)
Enzyme
0.05 N NaOH
50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*)
Burette

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1. Into each of six 25-ml Erlenmeyer flasks, place 10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) thymolphthalein indicator.

   This titration cocktail is used to quench the reactivity of subsamples of the reaction mixture. Six flasks are used for five time points plus a reagent blank.

2. Into a 50-ml Erlenmeyer flask with stopper, place 50 ml of 5% (w/v) olive oil/gum arabic emulsion substrate and preincubate 15 min in a 37°C water bath with magnetic stirring.

3. Add an appropriate amount of enzyme to initiate lipolysis on the emulsion substrate, start timer, and continue stirring.

4. At five suitable reaction intervals (e.g., 5, 10, 15, 20, and 25 min), remove 5 ml reaction mixture and transfer each subsample to a separate flask containing titration cocktail prepared in step 1. Swirl contents immediately to stop the reaction.

   The quenched subsamples may be turbid. Samples may be put aside (up to 2 to 3 hr at 20° to 22°C) for later titrimetric analysis.

5. Titrate the contents of each flask with 0.05 N NaOH using a burette until a light blue color appears.

   The pH indicator range of thymolphthalein is 9.3 to 10.5.

6. Into the last 25-ml Erlenmeyer flask containing titration cocktail, add 5 ml phosphate-buffered olive oil/gum arabic emulsion substrate and mix well. Using a burette, titrate the contents of this flask with 0.05 N NaOH to serve as a reagent blank.

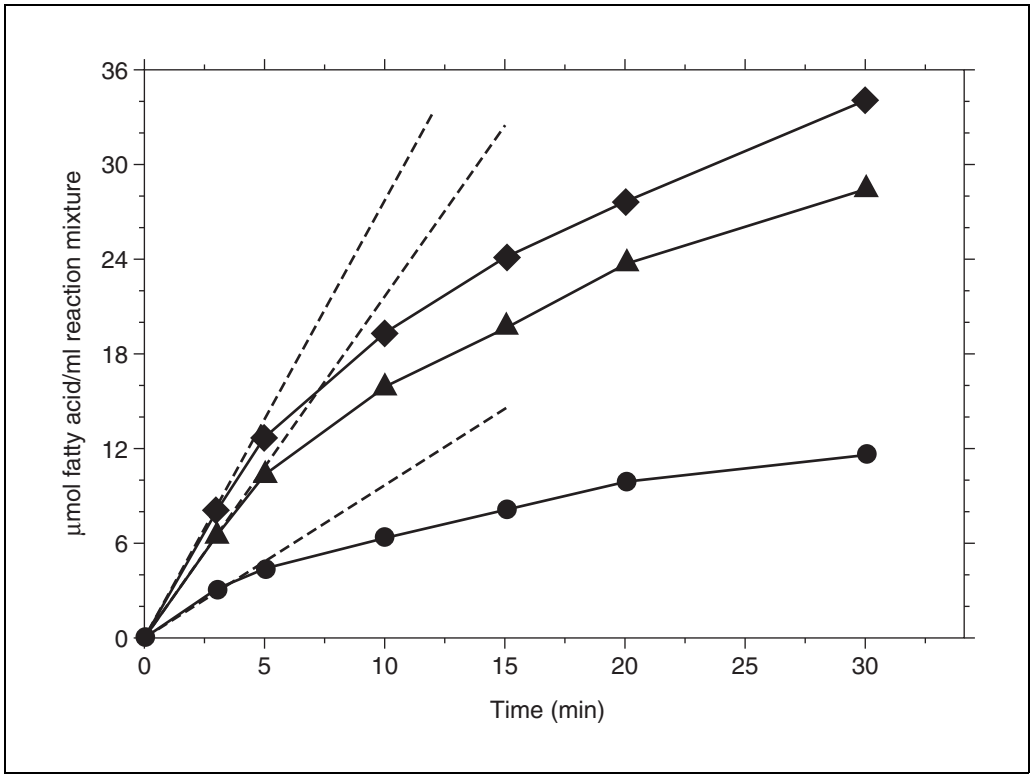

Figure C3.1.1 Comparison of lipase activities using the titrimetric assay. The lipases used in the reaction mixture, all at 0.1 mg/ml, were from *B. cepacia* (diamonds), *C. rugosa* (triangles), and porcine pancreas (circles). Broken lines represent estimation of initial reaction rates.

7. Calculate the quantity of fatty acids liberated in each subsample based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

   μmol fatty acid/ml subsample =

   [(ml NaOH for sample − ml NaOH for blank) × N × 1000]/5 ml

   where N is the normality of the NaOH titrant used (0.05 in this case).

8. Create a reaction progress curve by plotting the quantity of fatty acid liberated over the time of reaction (Fig. C3.1.1). Determine the activity (initial velocity, v_0) of the lipase from the slope of the linear portion (see Critical Parameters) using the following equation:

   v_0 = slope = $(y_2 - y_1)/(x_2 - x_1)$

   where units are μmol/(ml × min), equivalent to mM/min.

9. Determine the specific activity (sp. act.) of the lipase preparation, if the protein content of the added enzyme preparation is known, using the following equation:

   sp. act. = v_0 ÷ [a mg protein/(50 + b) ml reaction volume]

   where a is mg protein added in b volume to the reaction mixture, and units are μmol/(min × mg protein preparation).

   If a liquid form of the enzyme is added, such as lipases in broth samples from microbial cultures, calculate specific activity on a normalized basis by substituting a ml enzyme sample added (instead of mg protein) using the equation above.

10. Determine the number of units (U) of lipase activity, which is defined as the amount that produces 1 μmol of fatty acid per minute under the specified assay conditions.

    Depending on the nature of the original enzyme sample, specific activity can be expressed as U/mg protein or as U/ml for liquid forms of an enzyme of unknown concentration.

COLORIMETRIC ASSAY OF LIPASE ACTIVITY USING THE COPPER SOAP METHOD

BASIC PROTOCOL 2

Fatty acids liberated during hydrolysis of an olive oil substrate by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λ_{max} 715 nm), yielding a blue color. Quantification of fatty acid released by lipase is determined by reference to a standard curve prepared using oleic acid.

Materials

25 mM oleic acid standard solution
Benzene
Cupric acetate/pyridine reagent (see recipe)
Olive oil/Triton X-100 emulsion substrate (see recipe)
50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*)
Enzyme

15- to 20-ml screw-cap test tubes
Spectrophotometer (visible lamp)
Glass cuvettes

Prepare standard curve

1. Into ten individual 15- to 20-ml screw cap test tubes, place 0.1 to 1.0 ml of 25 mM oleic acid standard solution and dilute each to 5 ml with benzene for the standard curve (final 2.5 to 25 µmol oleic acid).

   CAUTION: *Benzene is toxic to humans, and is listed as a carcinogen by the United States Environmental Protection Agency. Handle with care, wear gloves, and work in a well-ventilated fume hood.*

2. Add 1 ml cupric acetate/pyridine reagent to each tube and vortex 2 min. Centrifuge 5 min at $1000 \times g$, room temperature.

3. Transfer a sufficient volume of the upper, clear benzene phase into a glass cuvette.

4. Measure the A_{715} of the benzene layer against a benzene blank and compose a standard curve by plotting A_{715} versus the amount of oleic acid in 5 ml benzene.

Perform lipase activity assay

5. Into each of seven 15- to 20-ml screw-cap test tubes, place 5 ml benzene and 1 ml cupric acetate/pyridine reagent.

   These reagents quench reactivity in subsamples of the reaction mixture. Seven tubes are used for six time points plus a reagent blank.

6. Prepare a reagent blank by adding 0.3 ml olive oil/Triton X-100 emulsion substrate to one of the tubes, vortex 2 min, and centrifuge 5 min at $1000 \times g$, room temperature.

7. Into a 50-ml Erlenmeyer flask with a stopper, place 25 ml olive oil/Triton X-100 emulsion substrate. Preincubate 15 min with magnetic stirring in a water bath set at 37°C.

8. Add a sufficient amount of enzyme (limit to 0.5 ml in solution form) to initiate lipolysis on the emulsion substrate, start timer, and continue stirring.

9. Remove duplicate 0.3-ml subsamples of the reaction mixture at predetermined time intervals (e.g., 4, 8, 12, 16, 20, and 30 min) and place in individual assay tubes prepared in step 5. Immediately vortex 2 min to stop the reaction and form the colored fatty acid cupric soaps. Centrifuge 5 min at $1000 \times g$, room temperature, to obtain the clear benzene upper phase.

   Samples are stable at this point for up to 3 days (Lowry and Tinsley, 1976) and can be set aside for later analysis.

10. Zero the spectrophotometer at 715 nm with the benzene layer obtained from the reagent blank. Measure the A_{715} for the benzene layer of each sample using glass cuvettes.

Analyze data

11. Convert A_{715} values to µmol oleic acid/ml subsample to yield mM concentration as follows:

    µmol fatty (oleic) acid/ml subsample =

    $(A_{715} - y \text{ intercept})/(\text{slope} \times 0.3 \text{ ml subsample})$

    In a typical assay, respective values of 0.0407 and 0.0433 are recorded for the y intercept and slope from the standard curve (see Anticipated Results).

12. Construct a reaction progress curve by plotting the concentration of oleic acid versus reaction time. Draw a tangent to the initial portion of the progress curve to obtain initial reaction rates (v_0 in mM/min) as follows:

v_0 = slope = $(y_2 - y_1)/(x_2 - x_1)$

13. Determine specific activity (sp. act.) of the lipase preparation by accounting for the level of protein (mg) in the added enzyme:

    sp. act. = $v_0 \div [a$ mg protein/$(25 + b)$ ml reaction volume]

    where a is mg protein added in b volume to the reaction mixture, and units are μmol/(min × mg protein preparation).

    Specific activity can be expressed in U (μmol/min) per mg protein, or as U/ml if the enzyme is in a liquid form and the concentration of protein is unknown. All calculations in this protocol are similar to those used in Basic Protocol 1. However, differences occur in the use of the standard curve, magnitude of volumes and quantities of subsamples, the reaction mixture, and level of enzyme added.

SPECTROPHOTOMETRIC DETERMINATION OF LIPASE ACTIVITY USING *p*-NITROPHENYL LAURATE AS SUBSTRATE

BASIC PROTOCOL 3

This method quantifies the level of *p*-nitrophenol (λ_{max} 400 to 410 nm) released following the hydrolysis of *p*-nitrophenyl laurate substrate by lipase. Activity of lipase can be calculated by comparing sample A_{410} values to those of a standard curve prepared with *p*-nitrophenol. *p*-Nitrophenyl laurate (and other carboxylic acid esters) are model or "synthetic" substrates. As with other model substrates, the ease of use of *p*-nitrophenyl acyl esters is balanced by providing only a presumptive test for lipase activity. However, this assay is often the method of choice for screening purposes or to provide an initial assessment of suitable assay conditions. Lipase is classified by the IUB Enzyme Commission as triacylglycerol acylhydrolase (EC 3.1.1.3) and, by definition, the native substrate is a triacylglycerol. Other lipolytic enzymes, and some proteolytic enzymes, may hydrolyze *p*-nitrophenyl acyl esters but be inactive toward triacylglycerols. Use of a shorter chain length *p*-nitrophenyl acyl ester, especially the water-soluble acetate derivative, would provide a preliminary indication of esterase-type activity of an unknown preparation.

Materials

0.5 mM *p*-nitrophenol standard solution (see recipe)
0.1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*)
420 μM *p*-nitrophenyl laurate substrate solution (see recipe)
Lipase solution

15- to 20-ml test tubes
Spectrophotometer
Cuvettes

1. Place 0.05 to 0.50 ml of 0.5 mM *p*-nitrophenol standard solution into ten individual 15- to 20-ml test tubes and dilute each to 5 ml with 0.1 M Tris·Cl buffer, pH 8.2.

   This yields a standard curve of 0.005 to 0.05 μmol p-nitrophenol/ml.

2. Measure A_{410} using 0.1 M Tris·Cl buffer, pH 8.2, as a blank, and make a standard curve by plotting A_{410} versus the *p*-nitrophenol concentration in each tube.

3. For each lipase activity assay, place 2.5 ml of 0.1 M Tris·Cl buffer, pH 8.2, and 2.5 ml of 420 μM *p*-nitrophenyl laurate substrate solution into a 15- to 20-ml test tube. Prepare one extra tube for a reagent blank.

4. Add 1 ml water to the reagent blank.

5. Add 1 ml lipase solution to the next substrate-containing tube to initiate the reaction. Start the timer, vortex briefly, and immediately transfer the reaction mixture into a cuvette. Record A_{410} every minute (or every 30 sec) for up to 15 min.

   For some spectrophotometers, continuous recording is available, and preferable. Some degree of automation may also be used if available.

6. Use the *p*-nitrophenol standard curve to convert absorbences to mM substrate hydrolyzed (see Anticipated Results) as follows:

   μmol *p*-nitrophenol/ml reaction mixture =

   (A_{410} − *y* intercept)/(slope × 6 ml reaction mixture)

   In a typical assay, respective values of 0.002 and 17.2 are recorded for the y intercept and slope from the standard curve (see Anticipated Results).

7. Determine lipase activity by constructing a reaction progress curve of concentration of *p*-nitrophenol (mM) released versus reaction time. Draw a tangent to the initial portion of the progress curve to obtain initial reaction rates (v_0 in mM/min) as follows:

   v_0 = slope = $(y_2 - y_1)/(x_2 - x_1)$

8. Determine specific activity (sp. act.) of the lipase preparation by accounting for the level of protein (mg) in the added enzyme:

   sp. act. = $v_0 \div$ [*a* mg protein/6 ml reaction volume]

   where *a* is mg protein added to 6 ml reaction mixture, and units are μmol/(min × mg protein), which is equivalent to U/mg protein.

   If the enzyme is in a liquid form and the concentration of protein is unknown, then U (μmol/min) can be expressed on the basis of ml instead of mg. All calculations in this protocol are similar to those used in Basic Protocol 2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cupric acetate/pyridine reagent

Place 5 g cupric acetate into a 100-ml volumetric flask and bring to volume with water. Swirl to dissolve, then filter through Whatman no. 1 filter paper. Adjust pH of the solution to 6.0 to 6.2 using pyridine. Store at room temperature (stable for a year or more).

p-Nitrophenyl laurate substrate solution, 420 μM

Place 0.0135 g *p*-nitrophenyl laurate (mol. wt. 321.4), 0.017 g sodium dodecyl sulfate (SDS), and 1.00 g Triton X-100 into a 100-ml volumetric flask and bring to volume with water. Heat the mixture in a water bath at 65°C for 15 min, mix well, and let the solution cool to ambient temperature prior to use. Store up to 3 days at 4°C. Reheat if the solution becomes turbid.

p-Nitrophenol standard solution, 0.5 mM

Place 0.0869 g *p*-nitrophenol (mol. wt. 139.1) in a 25-ml volumetric flask and bring to volume with 0.1 M Tris·Cl, pH 8.2 (*APPENDIX 2A*). Store up to one month in a tightly sealed vessel at room temperature. Dilute 1 vol with 49 vol of 0.1 M Tris·Cl buffer for a final 0.5 mM *p*-nitrophenol standard solution.

Olive oil/gum arabic emulsion substrate

Combine 10 g each olive oil and gum arabic in a 400-ml beaker. Bring volume to 200 ml with 50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*), and homogenize 5 min using a domestic blender or sonic-probe homogenizer at a setting that does not cause excessive foaming. Prepare fresh daily and rehomogenize periodically, or continuously subject the emulsion to low-speed magnetic stirring during an entire day of use.

Olive oil/Triton X-100 emulsion substrate

Weigh 5 g each olive oil and Triton X-100 in a 100-ml volumetric flask, and dissolve the mixture by adding 5 ml chloroform. Evaporate the chloroform under a stream of nitrogen gas at 60°C. This takes ~15 min, and the mixture becomes turbid. Slowly add warm (60°C) 50 mM sodium phosphate buffer, pH 8.0 (*APPENDIX 2A*), while swirling the flask and bring to 100 ml. Transfer the olive oil emulsion to a 250-ml beaker and homogenize 5 min using a homogenizer at a setting that does not cause excessive foaming. Let the emulsion stand at room temperature until most of the foam subsides. Adjust the final pH to 8.0 using 2 N NaOH. Prepare fresh daily and rehomogenize periodically, or continuously subject the emulsion to low-speed magnetic stirring during an entire day of use.

CAUTION: *Avoid exposure to chloroform. Use in a well-ventilated fume hood.*

COMMENTARY

Background Information

There are many procedures available for assaying or characterizing hydrolytic activity of lipases (Jensen, 1983; Vorderwülbecke et al., 1992; Schmid and Verger, 1998; Thomson et al., 1999). Owing to the nondistinct spectral nature of the products of triacylglycerol hydrolysis (fatty acids and partial glycerides), direct spectrophotometric analysis of reaction mixtures is not suitable for assessing lipase activity on native substrates. Consequently, most lipase assays have been developed on the basis of measuring liberated fatty acids either specifically or nonspecifically. Alternatively, the use of chromogenic or fluorogenic model substrates (analogs) affords the option to use spectrophotometry to directly and continuously follow the course of lipase (esterolytic) reactions. Other approaches are less commonly used and are based on sophisticated instrumentation and/or exploit the ability to measure physicochemical changes brought about by lipase action on native substrates and interfaces.

Titrimetric method

The most common method of nonspecific measurement of fatty acids is the titrimetric method (Basic Protocol 1). This is perhaps the simplest method, from both conceptual and procedural viewpoints (Benzonana and Desnuelle, 1968). Titrimetry can also be considered the benchmark method, since other assay methods, especially during the phases of development or modification, are routinely tested for how well they correlate to titrimetric assays. Fatty acids are weakly acidic, with pK_a values on the order of 4.7 to 4.9 for saturated 4- to 14-acyl carbon species, to 7.7 to 8.9 for oleic acid in the presence of 0 to 0.1 M Na^+ (linoleic acid has a pK_a of 7.9). The fatty acids liberated during the course of reaction can be determined quantitatively by titration using a standard alkali titrant and an appropriate end point.

Reactions are quenched by the addition of ethanol, which not only attenuates enzyme action but also facilitates solubilization of the fatty acids during subsequent titration to yield sodium salts (the presence of Ca^{2+} or Mg^{2+} can interfere in the assay by forming fatty acid soaps recalcitrant to titration). One will note a clearing of the titration vessel as titration progresses, even though the ethanol-quenched subsample may initially be turbid because of insoluble fatty acids. The choice of a thymolphthalein end-point (pH 9.3 to 10.5) indicator is essential to ensure the complete titration of all fatty acids, in view of the relative high pK_a values for oleic acid noted earlier.

The choice of the 50 mM sodium phosphate buffer at pH 8.0 is consistent with the optimal pH of lipases often being in the range of 7 to 9, although plant seed lipases are notable exceptions (Jensen, 1983). Phosphate provides buffering capacity over the pH range of 6.3 to 8.1

(pK_{a2} = 7.2) and helps maintain reaction pH as product (fatty acid) is formed. This buffer system contributes little to titratable acidity of quenched reaction mixtures ($pK_{a2/3}$ = 7.2/12.3), and the low background levels of titratable phosphate are easily accounted for in appropriate reagent blanks. This feature renders the titration step primarily responsive to newly evolved acidic species (i.e., fatty acids) in the reaction mixtures, conferring a large signal-to-noise ratio to this procedure. The Na^+ reduces the pK_a of oleic acid to a range that ensures complete titration. Thus, although the titration step itself is not specific to fatty acids, the manner in which the procedure is carried out affords the specificity necessary for the procedure to be accurate. Precision is afforded by careful preparation and assaying of reagent blanks. The coefficient of variation in analyses of lipase activity using titrimetry is on the order of 5% to 10% (Benzonana and Desnuelle, 1968; Hafkenscheid et al., 1983).

The titrimetric method also allows for virtually any triacylglycerol to be used as a substrate, as long as the substrate can be dispersed in liquid form; high-melting glycerides that partially solidify may cause anomalous results (Jensen, 1983). Because of this feature, titrimetry is a popular choice among these three methods for surveying or characterizing substrate selectivity by making use of pure or natural sources (e.g., vegetable oils) of glyceride substrates of different acyl chain compositions. In fact, assessment of lipolytic action on separate emulsions of monoacid triacylglcyerols (of 4 to 18 saturated acyl carbon lengths) and olive oil (or triolein) are often used to provide an initial indication of chain length selectivity for the enzyme (lipase) preparation of interest. Including an assessment of activity on triacetin (a water-soluble substrate) would expand the analysis to include an estimate of the balance of esterase and lipase activities in the preparation. The substrate tributyrin would lead to ambiguous results in this regard, because it has limited solubility and would exist as both soluble and interfacial forms in an assay system when included at levels above ~1% (solubility is also dependent on medium composition).

A pH-stat or automatic titration instrument greatly simplifies titrimetric analyses in that no buffer is required and the time course of tritrant addition may be available as a continuous recording (facilitating estimates of initial rates; see Critical Parameters). However, one limitation of the automatic titrator compared to the manual titration protocol is that not all fatty acids may be titrated if the pH-stat is set to maintain pH 7 to 8, making correction factors necessary for substrates evolving oleic or linoleic acids (Lowe, 1999).

Colorimetric method

Conceptually, assays for lipase activity using the colorimetric method (copper-soap procedure; Basic Protocol 2) are similar to titrimetry in that liberated fatty acids are being measured; however, the colorimetric method is more specific for fatty acids (Lowry and Tinsley, 1976). Quenched subsamples of emulsified acylglycerol/lipase reaction mixtures are combined with the biphasic mixture of cupric acetate/pyridine and benzene. Cupric salts of the fatty acids are formed (molar stoichiometry of fatty acid to Cu^{2+} of 4:2) and these soaps, which are blue in color, are partitioned into benzene to allow for quantification by measuring absorbance of the clear benzene phase at 715 nm.

The presence of pyridine is critical on two accounts. It appears to prevent cupric fatty acid salts from forming micellar aggregates of 40 or more fatty acids, which is important because the formation of these aggregates limits partitioning of cupric fatty acid salts into benzene. Consequently, sensitivity of the assay is increased by the inclusion of pyridine in the assay cocktail. Pyridine is also suspected of displacing water of hydration of the cupric ions of the soap, thereby enhancing copper soap solubility in the apolar phase (benzene). This not only increases sensitivity, but also normalizes the partitioning/solubility parameters of copper soaps of fatty acids of saturated 12- to 20-acyl carbons. This gives rise to a single response factor for these fatty acid species, although oleic acid gives a greater response because of its greater solubility as a cupric soap in organic media. Copper soaps of fatty acids less than 12 acyl carbons are progressively less soluble in benzene as acyl chain length is reduced, making sensitivity of the assay to these species variable, and less than is seen for longer chain length fatty acids. This requires correction factors to be obtained by the user in the event that 6- to 10-acyl chain length substrates are the subject of evaluation.

When identical lipase reaction subsamples are measured for fatty acid levels by both titrimetry (Basic Protocol 1) and colorimetry (Basic Protocol 2), estimates by the colorimetric procedure are only 60% of those obtained by titrimetry. However, the nature of this rela-

tionship may also be embedded in subtle differences between assay systems. When isooctane is used to replace benzene in Basic Protocol 2 to obviate the suspected toxicity of benzene, estimates are only 20% of that obtained by titrimetry (Kwon and Rhee, 1986). This indicates a semi-empirical nature to the copper soap procedure in that only a proportion of the cupric fatty acid soaps are partitioned into the organic layer. This partitioning is solvent dependent and compromises some degree of sensitivity of the assay. However, the results from the colorimetric and titrimetric procedures correlate highly (Hafkenscheid et al., 1983). The estimated coefficient of variation for results from the copper soap procedure is ~5% (Lowry and Tinsley, 1976). Another reagent that forms complexes with fatty acids is rhodamine B. However, this reagent is most often used to detect fatty acids on thin-layer plates or lipolytic activities on agar plates based on fluorescence of the fatty acid-dye complex (Kouker and Jaeger, 1987).

Spectrophotometric method

The most common spectrophotometric procedure used for lipase assays, and the one described here, is based on using the substrate analogs *p*-nitrophenyl acyl esters (Basic Protocol 3). The basis of this procedure is that lipases possess general esterolytic activity toward a variety of native and non-native carboxyl ester substrates. The *p*-nitrophenyl acyl substrate analogs are hydrolyzed to yield the fatty acid and *p*-nitrophenol, which is a chromophore (λ_{max} 400 to 410 nm) in slightly alkaline media (pK_a = 7.2) with a large extinction coefficient (ε_{mM} 18.3 at 400 nm and pH 10.2). Since *p*-nitrophenyl acyl esters may be hydrolyzed by nonspecific esterases in a given enzyme preparation, results with this type of substrate are only presumptive for lipase activity, and this would have to be confirmed by other assays using native substrates (such as Basic Protocol 1 or 2). Other less commonly used substrate analogs include β-naphthyl- (chromogenic) or umbelliferyl- (fluorogenic) acyl esters, or resorufin-linked acylglycerols (fluorogenic); assays based on their use are similarly presumptive for lipase activity (Jensen, 1983).

Despite the aforementioned limitation, the use of *p*-nitrophenyl acyl esters is the most sensitive of the three protocols described, and has an estimated coefficient of variation in the procedure of ~5%. It is essential for the user to prepare a standard curve for *p*-nitrophenol, because the pK_a and user-selected pH of the assay system may not afford complete spectrophotometric detection (i.e., ionization) of the released *p*-nitrophenol to *p*-nitrophenoxide.

This protocol is also suitable for assessing chain length selectivity of lipolytic-active fractions, as *p*-nitrophenyl acyl esters are readily available for acyl chain lengths of 2 to 18 (in fact, *p*-nitrophenyl laurate is not always the best analog for assaying all lipases). Activity toward the shorter chain length derivatives in this series of substrates, especially *p*-nitrophenyl acetate, is diagnostic for esterase activity. Both the spectrophotometric and titrimetric methods are advantageous over the colorimetric method in terms of assessing chain length selectivity of lipolytic enzymes. This is because the latter method requires complicated correction factors for measuring fatty acids released from monoacid triacylglycerols of 6 to 10 acyl carbon chain lengths, and lacks sensitivity in measuring lipase activity on monoacid substrates of shorter acyl chain lengths (acetic and butyric).

The greatest advantage of the spectrophotometric method is that it is direct and rapid, requires no sample workup, and allows for continuous assays of lipase activity compared to the multiple fixed-time-point analyses incumbent within Basic Protocols 1 and 2. The spectrophotometric method can also be done using very small volumes (as small as 1 ml) and is suitable for following the course of purification (such as in chromatographic fractions) or adaptable to 96-well plates (and subject to automation, if available). Thus, it is the method of choice for screening several samples or preparations for lipase (esterase) activity.

Choosing an assay method

In addition to assay features already mentioned, other factors may influence the choice of assay by the user. In terms of sensitivity of the assay, the threshold of detection of lipase activity, using the procedures as described in this unit, is on the order of 10^{-2} U for titrimetry, 10^{-1} U for colorimetry, and 10^{-4} U for spectrophotometry (where U is the amount of enzyme required to yield 1 μmol product per minute). The smallest amounts (volumes) of materials, including enzyme, are required for the spectrophotometric method, and progressively more material is required for the colorimetric and titrimetric methods. Unless a flow cell adapter is available, the spectrophotometric method is not suitable for analysis of particulate (immobilized) enzyme preparations, whereas the other assay procedures are.

Critical Parameters

Enzymes and assays

It is recommended that all enzyme assays be replicated three times. Powdered enzymes can be dissolved in assay buffer fresh daily and kept on ice prior to assay.

Substrate emulsion

All of the described procedures use emulsified substrate. Although the *p*-nitrophenyl laurate assay cocktail is stable for 3 days at 4°C, the emulsified olive oil substrates (or other triacylglycerol-based substrate systems) should be made fresh daily and rehomogenized periodically and when separation is visually evident. Use of day-old emulsion substrate will yield increased blank values for titratable acidity, and this effectively compromises the limit of detection of activity. Emulsified substrates should be in liquid form at common assay conditions (20° to 50°C), and partially solidified substrates (those rich in long-chain saturated fatty acids) will cause interfacial irregularities and confound the assessment of lipases in ways that cannot be accounted for.

Once a method for emulsifying the substrate is defined, it should be reproduced as carefully as possible from one day to the next, as this will help maintain a constant surface area-to-concentration ratio for the substrate preparation. The substrate concentrations listed for these protocols provide for saturating conditions in "fine" emulsions. In addition, the surfactants used in each case help maintain a constant surface area by promoting the formation of emulsion droplets of fairly discrete sizes. Each surfactant has a characteristic critical micelle concentration and aggregation state(s), and these properties will largely determine interfacial area for an emulsified substrate (even though it may not be known to, or easily determined by, the user). However, over time, coalescence of emulsion particles will occur, giving rise to reduced surface area to the point where reaction rates may become limited in part by substrate. In addition, the resulting heterogeneity caused by "creaming" of the emulsion may compromise the ability to procure representative subsamples for conducting the lipase assay. Replicate analysis of a given enzyme preparation should take place over the course of different days (different substrate emulsion

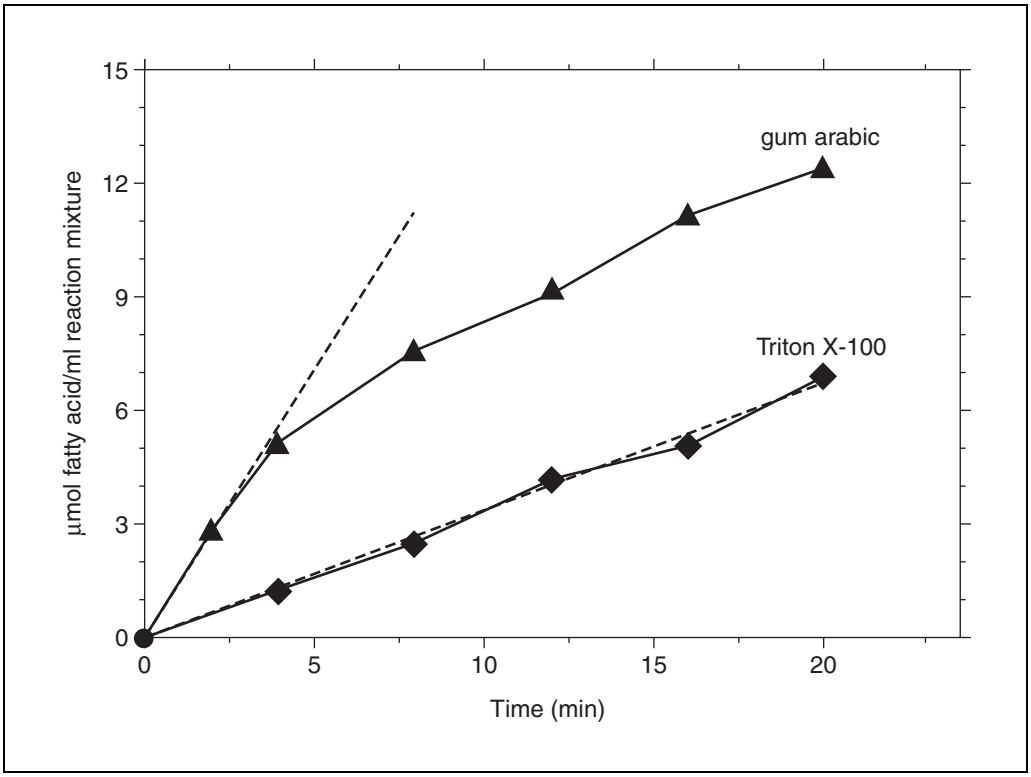

Figure C3.1.2 Comparison of lipase activities using an olive oil substrate emulsion prepared with different emulsifying agents. The lipase used in the reaction mixture was from *C. rugosa* (at 0.06 mg/ml) in the presence of 5% (w/v) each of gum arabic (triangles) or Triton X-100 (diamonds). Reaction progress analysis was obtained using titrimetry. Broken lines represent estimation of initial reaction rates.

preparations), as the quality and reproducibility of the substrate emulsion is arguably the most critical assay feature impacting on experimental variance (Lowe, 1999).

Measurement of initial reaction rates

Perhaps the most critical parameter the user should be concerned with, regardless of the protocol selected, is to ensure the measurement of initial reaction rates. To use someone else's words, "It must be stressed...that the activity of an enzyme is related to the initial rate of the reaction...rather than to the amount of product released...after an arbitrarily selected period of time" (Benzonana and Desnuelle, 1968). Too often in the scientific literature, activity measurements are made using a discontinuous, fixed-time-point approach without regard for verifying that true initial rates are being measured. The initial, linear portion can be rather easily identified using a continuous assay (such as in Basic Protocol 3), but requires more careful deliberation when using discontinuous or multiple-sampling-point procedures (such as in Basic Protocols 1 and 2). In either case, a tangent must be drawn to the initial portion of the progress curve that approximates (by linearization) the reaction rate initially observed (Figs. C3.1.1 and C3.1.2). Generally, greater enzyme activities lead to earlier departure from linearity of initial reaction rates and, in this case, improved results will likely occur if the enzyme is assayed again at a more dilute level, as illustrated in Figure C3.1.3. It is also important to avoid any lag (pre-steady-state) period in estimating initial reaction velocities, as this phenomenon is sometimes encountered. Curvilinear progress curves result from the tendency of liberated fatty acids to partition at the interface and block (inhibit) further access of enzyme to substrate, or possibly to block enzyme interfacial inactivation during reaction. Increasing the rigor of agitation of the reaction mixture may help prolong linear rates by mitigating product inhibition.

Anticipated Results

Analysis of three lipase reactions using the titrimetric method illustrates typical reaction progress curves and how, as well as the need, to estimate initial rates by tangential analysis (Fig. C3.1.1). The corresponding initial reaction velocities were 27.5 U/mg for *Burkholderia cepacia* (formerly, *Pseudomonas cepacia*) li-

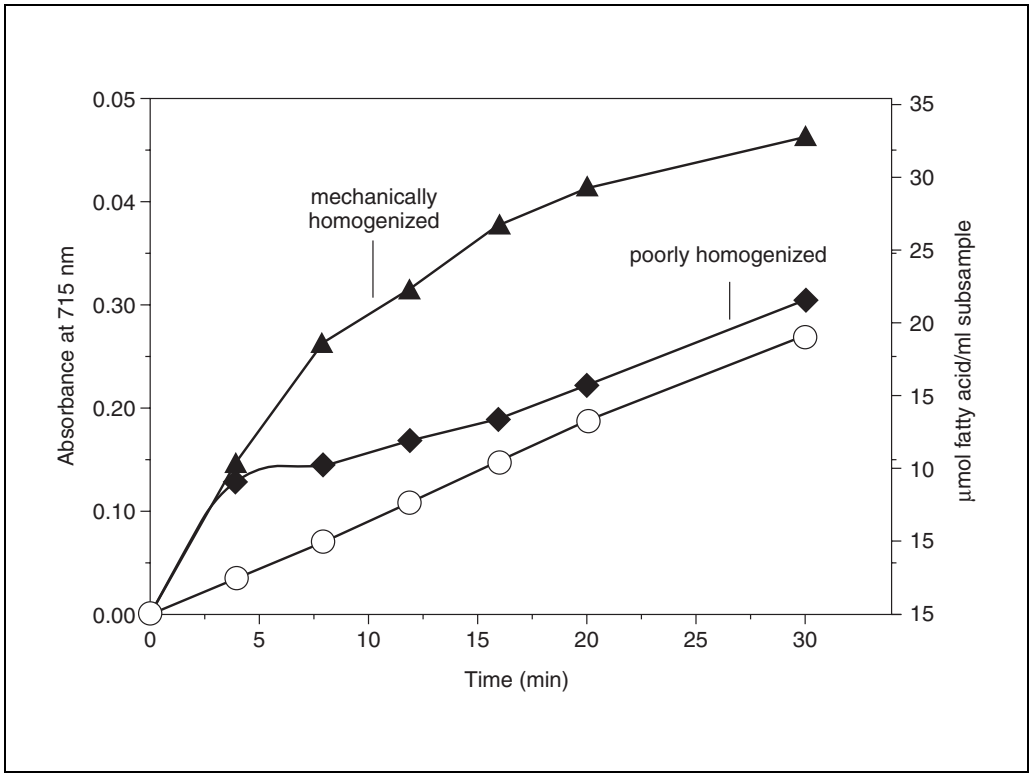

Figure C3.1.3 Comparison of lipase activities using the copper soap colorimetric assay as affected by the degree of homogenization of olive oil substrate. The lipase used in the reaction mixture was from *C. rugosa* (at 0.40 mg/ml) in the presence mechanically homogenized (triangles) or poorly homogenized (shaken by hand; diamonds) substrate emulsion. For comparison of linearity of reaction progress curves, the time course of *C. rugosa* lipase (at 0.20 mg/ml; circles) is provided.

pase, 21.8 U/mg for *Candida rugosa* lipase, and 9.70 U/mg for porcine pancreatic lipase. When Triton X-100 replaced gum arabic (gum acacia) in the emulsion system, the corresponding initial reaction rates for these same enzymes were 14.6 U/mg, 5.20 U/mg, and not detectable, respectively (data shown only for *C. rugosa* lipase in Fig. C3.1.2). These results indicate that the choice of surfactant influences lipase action either by modulating the nature or interfacial area of the dispersed phase and/or by direct influence on the lipase. Gum arabic also offers greater stabilization of the emulsion than does Triton X-100, and has evolved as the surfactant of choice when using titrimetric assays for lipase activity.

A direct comparison of the titrimetric and copper soap methods using olive oil emulsion and Triton X-100 as surfactant for *B. cepacia* lipase gave results of 14.6 U/mg and 5.32 U/mg, respectively, and 5.20 U/mg and 2.67 U/mg for *C. rugosa* lipase, respectively (Figs. C3.1.2 and C3.1.3). This illustrates the lower estimate obtained with the copper soap method compared to the titrimetric method. Triton X-100 is used as surfactant with the copper soap method because gum arabic forms a viscous emulsion in the presence of benzene during the sample workup steps. By the same token, if the user selects to substitute isooctane for benzene in this method (to avoid the toxicity of benzene), gum arabic is preferred over Triton X-100 as the surfactant, because Triton X-100 forms a viscous emulsion during sample workup in the presence of isooctane. A typical standard curve for oleic acid when using the copper soap method was: μmol oleic acid = 0.0433 A_{715} + 0.0407 (r^2 = 0.996).

The importance of the physical nature (often termed interfacial quality) of the substrate on the progress of assays for lipase activity is illustrated in Figure C3.1.3. Formation of fine emulsions by systematic, mechanical processes yields more typical progress curves (which are easier to analyze for initial reaction rates) than when using nonmechanically homogenized (coarse) emulsion, using *B. cepacia* lipase as an example. Even though initial reaction rates with the two substrate preparations may be similar, the reaction progress with the less-well-dispersed substrate can compromise or obscure the ability to make accurate estimates of initial reaction rates. The results with the "poorly homogenized" substrate emulsion are qualitatively similar to what would be observed if the emulsion was used throughout the day with evidence of visible phase separation and without periodic rehomogenization.

In the event that lipase preparations are too active to allow for facile estimation of initial rates, the enzyme can be diluted and assayed again. This is illustrated using the copper soap method where the reduced level of *C. rugosa* lipase addition afforded a longer period of linearity to the reaction progress curve than did the more active *B. cepacia* lipase (Fig. C3.1.3).

Assays using the spectrophotometric method (*p*-nitrophenyl acyl esters) are typically linear for 5 to 15 min depending on the levels of lipase activity. A typical standard curve for *p*-nitrophenol under the assay conditions described in this unit (pH 8.2) is: mM *p*-nitrophenol = 17.2 A_{410} + 0.002 (r^2 = 0.999). Results can range widely for different lipase preparations (Vorderwülbecke et al., 1992). For example *C. rugosa* and porcine pancreatic lipase preparations exhibited initial reaction rates of 1.39 U/mg and 0.0007 U/mg, respectively, using this assay method. Although these initial reaction rates are less than those observed using the assay systems designed for titrimetric and colorimetric analysis, the fact that such low activities can be measured underscores the sensitive nature and utility of the *p*-nitrophenyl acyl ester assay system, especially for screening dilute or crude preparations containing lipase.

Time Considerations

Aside from the time required to prepare reagents, the least amount of time is required per lipase assay by the spectrophotometric method, and the greatest amount of time is required per assay for the titrimetric method. Although all assays are described as requiring up to 30 min for the reaction mixture to be subsampled, time savings can be realized by subsampling more frequently over a shorter period of time, as long as one obtains valid initial rate data. Thus, for all assays, the time involved to run the lipase reaction can be normalized to be the same at ~10 to 15 min. The difference in time requirements for the protocols becomes embedded in sample workup procedures.

For the spectrophotometric method, there is no sample workup, allowing one to run ~4 assays/hr. This can be increased to ~16 to 100 or more samples/hr depending on equipment features and automation, such as multiple cuvette holders/changers and 96-well microplate readers. For the colorimetric procedure, sample workup requires ~10 min/subsample, but several samples can be "batch processed" simulta-

neously. Thus, one can comfortably complete 2 to 4 lipase activity determinations/hr, depending on how efficiently the user coordinates subsampling and sample workup. The titrimetric assay poses the greatest demands on time, as each subsample requires detailed attention during the titration step. One can reach the point of completing at most 2 activity determinations/hr, although this can be almost doubled using an automatic titrator or pH-stat instrument. The manual titration method is tedious and much more subject to user fatigue than the other protocols described in this unit.

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Key References

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Detailed account of kinetics and inhibition of pancreatic lipase action on emulsified triacylglycerol, including special treatment of initial rate measurements and pK_a of liberated fatty acids.

Jensen, 1983. See above.

Contrasts several popular and less common methods for detecting and assaying lipase activity. Discusses advantages, disadvantages, and the unique requirements of each method for obtaining accurate estimations of lipase activity.

Lowry and Tinsley, 1976. See above.

Describes a modified copper soap that is still in widespread use. Focus is placed on the development of solvent systems that simplified the procedure and enhanced sensitivity, and on issues relating to reproducibility.

Vorderwülbecke et al., 1992. See above.

An exhaustive account of 73 commercially available lipase preparations, comparing activities using six hydrolytic and five esterification assay substrate systems. Illustrates the wide range of activities between lipases and for a given lipase assayed by different protocols.

Contributed by Praphan Pinsirodom and Kirk L. Parkin
University of Wisconsin
Madison, Wisconsin

Polarographic and Spectrophotometric Assay of Diphenol Oxidases (Polyphenol Oxidase)

The diphenol oxidases (DPOs) includes two major groups of enzymes: the ortho-DPOs (also known as catecholases, polyphenol oxidases, and tyrosinases) and the para-DPOs (more usually known as laccases). The names catecholase and laccase are used in this unit.

Both of these enzymes catalyze the oxidation of *ortho*- and/or *para*-diphenols to their respective quinones, which may subsequently react non-enzymically through a chain of reactions to form dark brown to black polymeric melanoidins. This is the typical sequence of reactions observed during the browning process that occurs when many fruits, other plant tissues, or Basidiomycete fungi (e.g., mushrooms) are cut or damaged in any way. Consequently, these enzymes are of great interest to food biochemists and technologists, and much effort has been and is devoted to methods of controlling enzymic browning. Some *o*-DPOs can also carry out hydroxylation reactions (e.g., tyrosine to 3,4-dihydroxyphenylalanine; hence the origin of the name "tyrosinase"). The basic reactions involved are summarized in Figure C4.1.1.

Two principal methods are widely used for the assay of DPOs. For enzyme kinetic studies, the most appropriate methods are polarographic and use an O_2 electrode (Basic Protocol), which allows the direct measurement of the rate of utilization of oxygen and a true comparison of different phenolic substrates. Minor disadvantages of this method are that it requires more specialized equipment and that assays can only be carried out one at a time. Nevertheless it has proven to be the basis of some excellent undergraduate biochemical laboratory experiments.

The other major types of assay are all spectrophotometric (Alternate Protocols 1 and 2) and usually involve measuring the production of the highly colored end-product of the DPO reaction. They are used widely because they are rapid, can be carried out with the simplest of colorimeters/spectrophotometers, and can be adapted to use microtiter plates (Alternate Protocol 3). Thus they are ideal for simple routine comparative or monitoring studies. A major weakness of these colorimetric procedures is that they measure the end-product of a complex sequence of reactions, and different substrates yield different

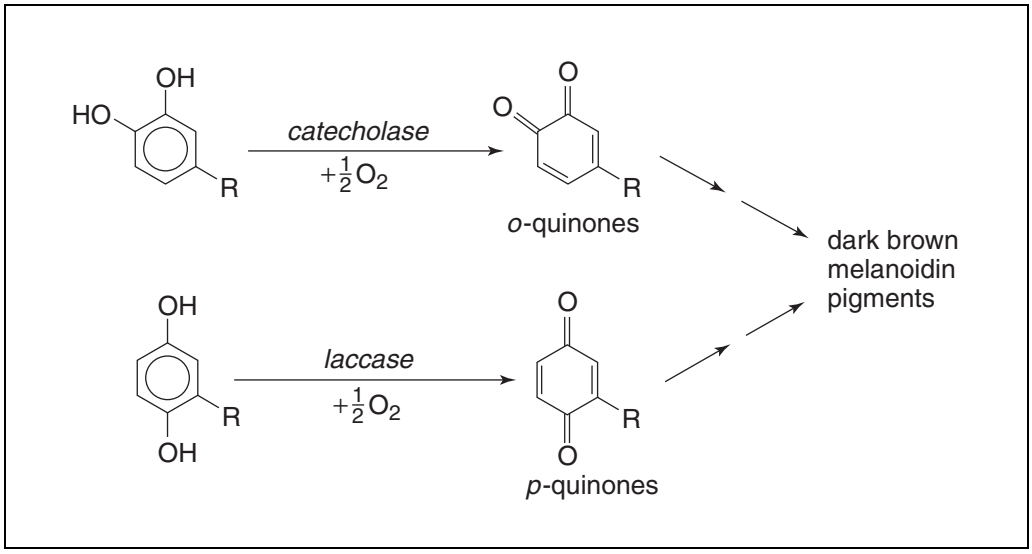

Figure C4.1.1 The basic reactions catalyzed by diphenol oxidases (DPOs).

Contributed by John R.L. Walker
Current Protocols in Food Analytical Chemistry (2001) C4.1.1-C4.1.15

final colors so that valid kinetic comparisons between substrates are not possible. Consequently simple spectrophotometric assays are less reliable for biochemical studies of enzyme kinetic parameters or substrate specificity.

Catecholases and laccases may be distinguished by substrate specificity tests and by the use of selective inhibitors. Another factor to be considered when investigating these enzymes is that many plant catecholases exist in latent or bound forms, so that full activity may not be expressed until the enzyme preparation is treated with a suitable chaotropic agent such as sodium dodecyl sulfate (SDS).

BASIC PROTOCOL

POLAROGRAPHIC ASSAY OF DIPHENOL OXIDASES

This unit describes the use of the Rank inverted O_2 electrode (Rank Bros.) for the assay of DPOs; however, this protocol is equally applicable to other types and brands of O_2 electrode (e.g., Oxygraph, Yellow Springs Instruments). The O_2 electrode is a simple device for the continuous measurement of O_2 activity (pO_2) in solution (Figure C4.1.2). Thus, it is ideal for the assay of any reaction that consumes or evolves O_2 (e.g., photosynthesis) and is particularly useful for the measurement of rapid reactions.

It is usually important to optimize the working concentration of enzyme in any assay system to ensure that the assay system gives a true measure of the initial rate of reaction (see Chapter C1 and Figure C4.1.3), and that the relationship between enzyme concentration and rate lies within the linear range. This is particularly important for O_2-electrode assays because the electrodes are separated from the biological reaction medium by a thin PTFE or polyethylene membrane that is permeable to O_2, and the diffusion of O_2 across the membrane can become rate limiting. It is also important in spectrophotometric assays. This process of optimization is called range-finding and is outlined in the protocol below.

This protocol is amenable to addressing several additional questions regarding DPO activity. Many plant *o*-DPOs are often present in a latent form (probably membrane-bound) and their activity may be enhanced in the presence of anionic detergents (chaotropic agents). The measurement of latent DPOs using SDS is outlined below. Additionally, the assay can be used to study the effect of DPO inhibitors. Finally, the assay can be used to study kinetic parameters of DPO activity. The O_2 electrode is the method of choice for the determination of enzyme kinetic parameters for DPOs since, unlike colorimetric assays, it measures the actual initial rate of O_2 uptake. These parameters include Michaelis constants (K_M) and inhibitor constants (K_i); for a full discussion of these aspects readers should refer to any standard textbook of biochemistry.

Materials

Oxygenated buffer (see recipe)
Saturated sodium dithionite solution, prepared fresh
Oxygenated water (air-saturated)
Sample enzyme extract
10 mM substrate solution (Sigma or Fluka; Figure C4.1.4), prepared fresh daily in water:
  For catcholases: 4-methylcatechol or catechol
  For laccases: 1,4-dihydroxy-2-methylbenzene (toluquinol) or 1,4-dihydroxybenzene (quinol)
1% (w/v) sodium dodecyl sulfate (SDS; optional)
Enzyme inhibitor(s) (optional)

O_2 electrode with magnetic stirrer, water-jacketed cell held at 30°C, temperature-controlled circulator, and data recording system (e.g., strip chart recorder with 1 or 10 mV input)
1-ml syringes with long (50-mm) ground needles

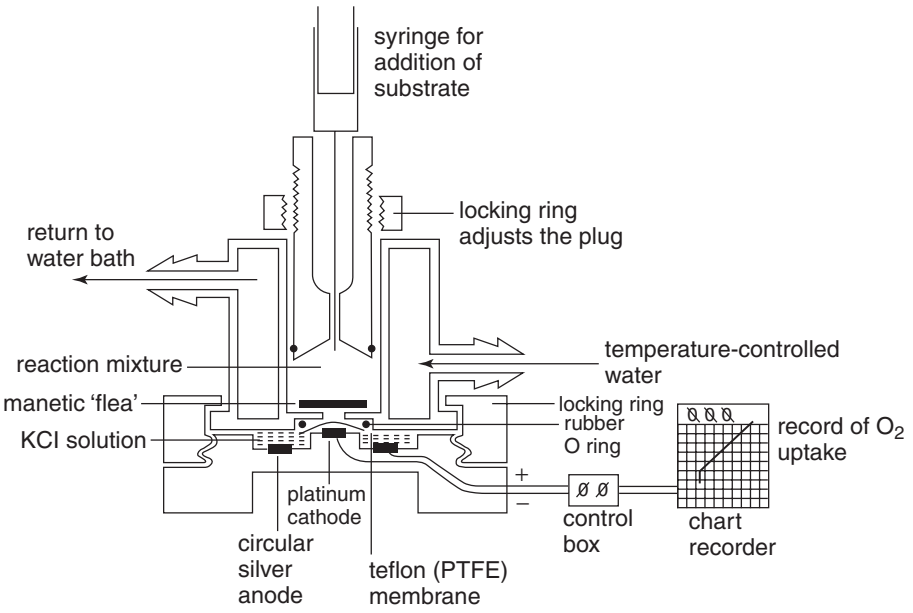

Figure C4.1.2 Diagram of the Rank inverted O_2 electrode.

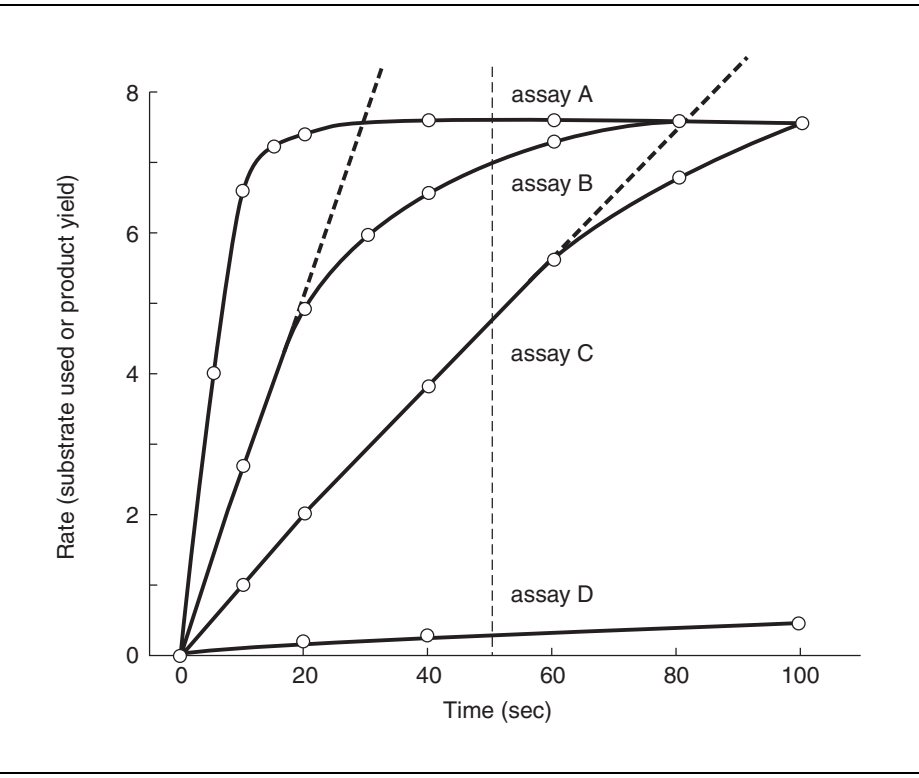

Figure C4.1.3 Range-finding and the effect of enzyme concentration on the time course of a reaction. The dotted lines show the true initial rate. Only assay C will be reliable if a fixed-time assay is used.

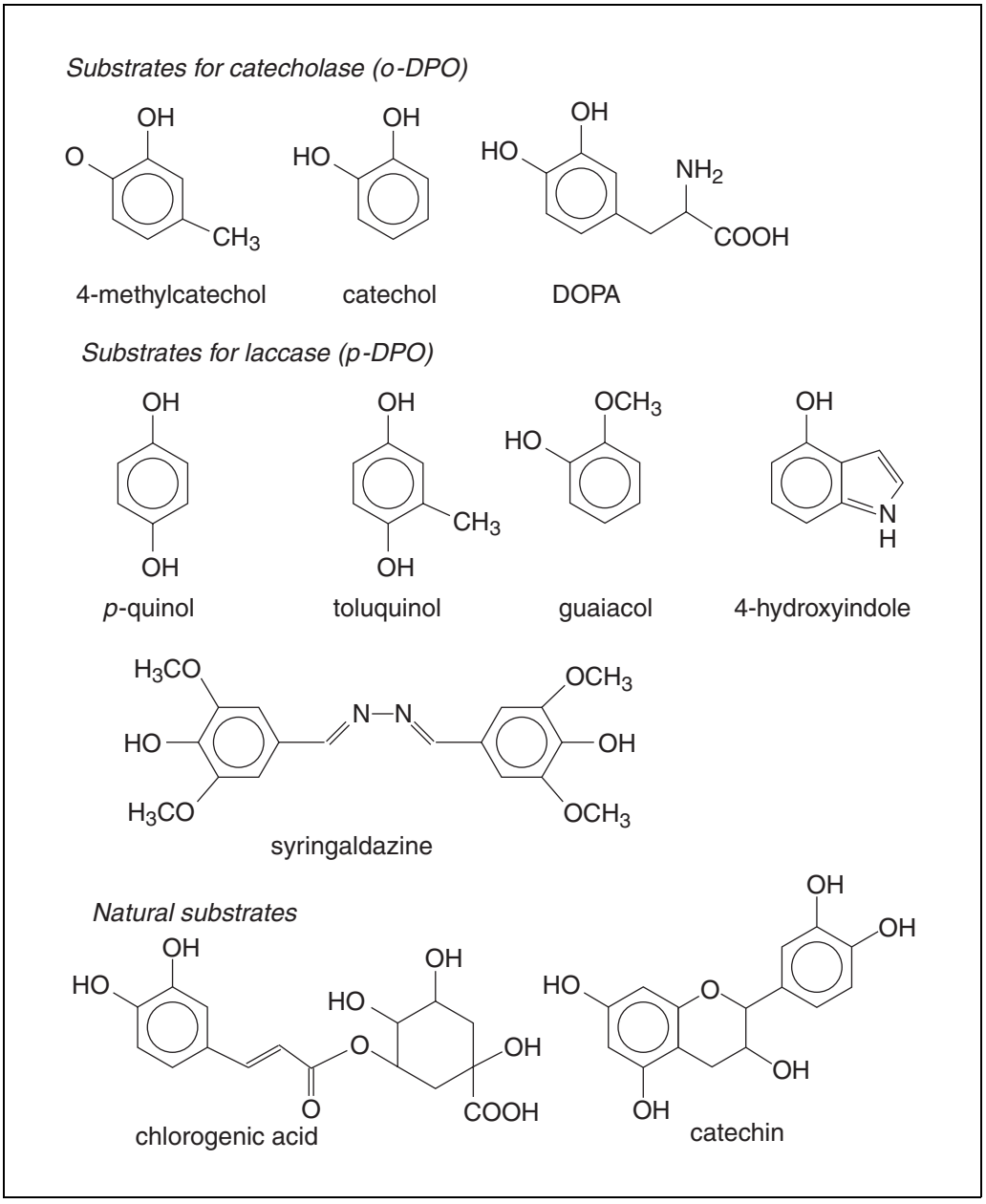

Figure C4.1.4 Structures of some common substrates for DPOs.

Set up electrode

1. Set up an O_2 electrode, control box, and data recording system according to the manufacturer's instructions.

   This protocol assumes the use of a strip chart recorder (1 or 10 mV input), but other means of continuous data recording could be used.

   When setting up an O_2 electrode it is important to make sure that there is a thin film of KCl electrolyte between the membrane and the Pt cathode. One way to ensure this is to place a small piece (~10 mm square) of single-thickness facial tissue (paper handkerchief) between the cathode and the membrane; this will prevent the membrane from being stretched too tight and excluding the electrolyte. The author has found readily available kitchen polyethylene plastic wrap to be an excellent material for O_2 electrode membranes.

2. Set the water circulation thermostat system to 30°C and allow system to equilibrate.

Because O_2 diffusion through the membrane is markedly affected by temperature, it is most important that the cell be kept at a constant temperature.

3. Place 2.5 ml oxygenated buffer in the electrode cell, replace the adjustable plug, and set its depth to ensure that no air bubbles are trapped beneath the plug. Switch on the magnetic stirrer.

   Because O_2 is consumed by the electrode reaction, it is essential to keep the solution stirred rapidly and thus avoid a localized O_2-depleted layer at the membrane.

4. To check the electrode response and set zero oxygen concentration, inject 0.5 ml freshly prepared saturated sodium dithionite solution through the hole in the cell plug (the recorder pen should move rapidly toward zero). Use this reading to set the zero baseline.

   If the electrode response is sluggish, first check/replace the membrane since most O_2 electrode problems are due to dirty or leaky membranes. Remove the cell plug and rinse the cell thoroughly with distilled water using a plastic disposable pipet to avoid the risk of damage to the membrane.

   It is essential that all traces of sodium dithionite be removed since it is a potent inhibitor of DPOs.

   Do not allow the membrane to become dry between experiments; always have some liquid in the cell.

5. Refill the cell with air-saturated water; use this as the 100% (fully saturated) setting and adjust the recorder pen to 95% full-scale deflection.

   If 100% O_2 is assumed to be equivalent to 233 μmol dissolved O_2 per liter (the solubility of O_2 in water equilibrated with air at 30°C), and if the cell contains a total of 3 ml liquid, then the total O_2 content = 3 ml × 233 μM = 699 nmol O_2, and one scale division corresponds to 7.4 nmol O_2. Consequently, rates of enzyme activity can be expressed as nmol O_2/min.

   In practice, especially for simple comparative studies, it is usually sufficient (and saves a lot of time) to set the chart recorder at 95% deflection when the cell is filled with O_2-saturated water and express rates of O_2 uptake in arbitrary units as ΔmV/min.

Perform range-finding to determine suitable enzyme concentration

6. Add 1.5 ml oxygenated buffer followed by 0.5 ml water and 0.5 ml enzyme extract to electrode chamber.

7. Replace cell plug and check for absence of air bubbles.

8. Switch on magnetic stirrer and allow 1 to 2 min to equilibrate. Adjust the chart recorder to read between 90% and 100% full scale.

9. Switch on the chart drive and allow a stable 100% baseline to be established.

   Satisfactory chart speeds usually range from ~50 to 100 mm/min (2 to 4 in./min).

10. Inject 0.5 ml of 10 mM substrate solution using a 1-ml syringe with long (50-mm) ground needle. Take care not to damage the membrane.

    If the enzyme is active, the recorder pointer should begin to move steadily towards zero.

    Substrates should be prepared fresh daily and discarded if they show any signs of discoloration due to autooxidation. 4-Methylcatechol usually gives the highest rate of O_2 uptake in catecholase assays, and toluquinol commonly gives the highest rate for the assay of laccases. It is important that the substrate be added last.

    It is a good idea to grind the points off the needles to minimize the risk of damage to the electrode membrane.

11. Monitor changes for 2 to 3 min and then switch off the chart drive to conserve paper. Empty cell and rinse thoroughly with distilled water.

12. Calculate the initial rate of O_2 uptake (as Δnmol O_2/min or ΔmV/min) from the slope of the line on the chart.

13. Repeat steps 6 to 12 using the volumes of enzyme indicated below.

Oxygenated buffer (ml)	1.5	1.5	1.5	1.5
Water (ml)	0.6	0.7	0.8	0.9
Enzyme (ml)	0.4	0.3	0.2	0.1
Substrate (ml)	0.5	0.5	0.5	0.5

For all subsequent experiments use the concentration of enzyme that gives 80% O_2 uptake within 40 to 80 sec, i.e., a plot with a slope of ~45° to 60° (Figure C4.1.3).

Measure DPO activity

14. Perform all subsequent assays as above, using the appropriate volumes of enzyme and water.

The final volume of solution must always add up to 3 ml.

Measure latent DPO activity (optional)

15. To determine whether increased rates of O_2 uptake can be observed due to the presence of DPO in a latent form, repeat steps 6 to 12 using the optimized enzyme concentration and adding 0.1 ml of 1% SDS to the enzyme assay mixture. Reduce the volume of water added by 0.1 ml to maintain a total volume of 3 ml.

Determine effect of inhibitors (optional)

16. Determine the effect of enzyme inhibitors by adding between 0.1 and 0.5 ml inhibitor solution at the required concentration and adjusting the amount of water to maintain the final total volume at 3 ml.

A typical inhibitor assay protocol might contain 1.5 ml buffer, 0.4 ml enzyme, 0.4 ml water, 0.2 ml inhibitor, and 0.5 ml substrate.

Determine enzyme kinetic parameters (optional)

17. To determine K_M values, conduct rate measurements (v) with at least four different concentrations of any given substrate, [S], and then analyze the data by any suitable kinetic plot such as the Lineweaver-Burke plot or the direct-linear plot (refer to any standard textbook of biochemistry for more information).

18. To determine inhibitor constants (K_i), repeat step 17 in the presence of one or two different concentrations of inhibitor, [I]. Alternatively, for a Dixon Plot, test a range of inhibitor concentrations at two different substrate concentrations. Then plot 1/v against [I] for each value of [S].

Computer programs (e.g., ENZPACK; Biosoft) are available to facilitate the plotting and statistical analysis of these results.

SPECTROPHOTOMETRIC ASSAY OF CATECHOLASE ACTIVITY

ALTERNATE PROTOCOL 1

The simplest, but less accurate, method of assaying DPO activity is to record the final color yield when the enzyme is incubated with a suitable phenolic substrate such as catechol, 4-methylcatechol, *tert*-butylcatechol, or 3,4-dihydroxyphenylalanine (Mayer et al., 1966). As with the polarographic assay system described in the Basic Protocol, it is advisable to establish an optimum working concentration of enzyme (range-finding; Figure C4.1.3).

As with the Basic Protocol, this method can be used to assess latent DPO activity by addition of SDS. This simple assay system may also be used to investigate the effect of DPO inhibitors by adding suitable quantities of test compound dissolved in buffer (Ferrar and Walker, 1996, 1999). However, it must be remembered that, in this assay system, the prevention of color development (enzymic browning) may be due to chemical reactions between the inhibitor and the reaction products preventing the formation of the colored end-product, rather than the actual inhibition of the enzyme. This problem may be avoided by use of the O_2-electrode assay (see Basic Protocol).

Additional Materials *(also see Basic Protocol)*

Buffer suited to enzyme being investigated, e.g., 0.2 M phosphate (KH_2PO_4/Na_2HPO_4) or citrate/phosphate (McIlvaine's) buffer, pH 4.0 to 6.5 (*APPENDIX 2A*)

10 mM substrate (Sigma or Fluka) solution (Figure C4.1.4) in buffer:
- 3,4-Dihydroxyphenylalanine (DOPA) (prepared fresh)
- 4-methylcatechol
- Catechol
- *tert*-Butylcatechol

Spectrophotometer or colorimeter with appropriate cuvettes or test tubes

Perform range-finding to determine suitable enzyme concentration

1. Switch on the spectrophotometer/colorimeter and allow to stabilize. Set the wavelength to 475 nm or use a blue filter.

2. Set up the following series of test tubes or cuvettes.

Buffer (ml)	1.5	1.5	1.5	1.5	1.5
Water (ml)	0.0	0.1	0.2	0.3	0.4
Enzyme (ml)	0.5	0.4	0.3	0.2	0.1

If this procedure is used with turbid or particulate enzyme preparations, it may be necessary to clarify the assay mixture by centifugation before reading the absorbance. Use of the O_2 electrode assay (Basic Protocol) avoids this problem.

3. Zero the spectrophotometer against a blank containing all reagents except the substrate.

4. Prepare a duplicate set of tubes containing an additional 1.0 ml water to serve as zero blanks (no substrate) for each concentration of enzyme.

5. Initiate the enzyme reaction by adding 1.0 ml of 10 mM substrate solution and mixing rapidly by stirring or by covering the cuvette with thin plastic film and inverting.

6. Monitor the increase in dopachrome (or other colored end-product) by recording the change in absorbance at 475 nm (ΔA_{475}) at 20- or 30-sec intervals.

The initial rate (v) of the reaction is expressed as $v = \Delta A_{475}$/min.

Because dopachrome is subject to autooxidation and polymerization reactions, the rate measurements should not be continued past 3 min. Similarly, the enzyme extract should be

suitably diluted if the rate of formation of dopachrome is too rapid (e.g., ΔA_{475} should take ≥2 min to reach 1 absorbance unit). If these constraints are not observed, the assay system may not obey Michaelis-Menten kinetics; refer to Figure C4.1.3.

Measure DPO activity

7. Perform all subsequent assays using the appropriate volumes of enzyme and water.

Once an optimum working concentration of enzyme has been established, it is sufficient to record the A_{475} after 60 sec and take this as the measure of activity.

Measure latent DPO activity (optional)

8. Test for latent DPO activity by repeating the above assay with the addition of 0.1 ml of 1% SDS to the assay mixture.

Determine effect of inhibitors (optional)

9. Determine the effect of enzyme inhibitors by repeating the above assay with the addition of inhibitor solution at the required concentration.

ALTERNATE PROTOCOL 2

SPECTROPHOTOMETRIC ASSAY OF LACCASE ACTIVITY

Many edible Basidiomycete fungi (e.g., mushrooms) and some fruits contain laccases. These may be measured spectrophotometrically in the same way as catecholases (see Alternate Protocol 1) by using laccase-specific substrates: 10 mM 4-hydroxyindole (Cai et al., 1993), 4 mM syringaldazine (Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981), 10 mM toluquinol, or 10 mM guiacol. The spectrophotometer should be set at 615 nm for 4-hydroxyindole, 525 nm for syringaldazine, or 452 nm for guiacol and toluquinol. The buffer should be 0.2 M phosphate (KH_2PO_4/Na_2HPO_4), pH 6.5. 4-Hydroxyindole and/or syringaldazine are the substrates of choice since they yield distinctive bright blue or bright mauve products; however, it should be noted that laccases from higher plants do not always react with syringaldazine (Harvey and Walker, 1999).

ALTERNATE PROTOCOL 3

MICROTITER PLATE ASSAYS OF DPOs

For rapid routine analyses of soluble DPOs, the spectrophotometric assays described above may be adapted for use in a microtiter plate reader, which offers considerable savings in time and materials (Ferrar and Walker, 1996, 1999). However, this type of assay is not suitable for very turbid solutions. As in the previous assays, it is advisable to establish an optimum working concentration of enzyme (range-finding; Figure C4.1.3). The recommended buffer is adjusted to a compromise pH that will work in most catecholase and laccase assays. This protocol should also be adaptable for assessing latent activity and affects of inhibitors.

Additional Materials *(also see Alternate Protocols 1 and 2)*

Buffer: 0.2 M phosphate (KH_2PO_4/Na_2HPO_4), pH 6.0
Enzyme sample dissolved in phosphate buffer
Microtiter plate reader and plates

1. Program the microtiter plate reader according to the manufacturer's instructions. Select the wavelength/filter(s) appropriate to the chosen enzyme and substrate as listed below:

DOPA or 4-methylcatechol: 452 nm
4-hydroxyindole: 615 nm
Syringaldazine: 525 nm
Toluquinol or guaiacol: 452 nm.

2. Set up the following series of test wells.

Buffer (µl)	150	150	150	150	150
Water (µl)	0	10	20	30	40
Enzyme (µl)	50	40	30	20	10

3. Add 50 µl substrate to start the reaction. Mix by swirling or use the built-in agitation program if included in the plate reader.

   Control blanks without added substrate should also be included.

4. Incubate at 30°C and read the absorbances at 2-min intervals for 10 min. Plot absorbance against time, as in Figure C4.1.3, to establish the optimum working concentration of enzyme.

5. Use this amount of enzyme plus water for all subsequent assays.

   Once a suitable working level of enzyme has been established, and for simple comparative tests, it is sufficient to record the ΔA after 10 min incubation and use this as a measure of activity. In this case, a unit of DPO activity may be arbitrarily defined as that concentration of enzyme giving a ΔA of 0.001 AU/min.

REAGENTS AND SOLUTIONS

*For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Oxygenated buffer

Hold buffer at 30°C and aerate with a stream of bubbles via a fine capillary tube. An aquarium aerator is a convenient way to achieve this. Select the buffer and pH to suit the enzyme being investigated. Citrate/phosphate (McIlvaine's) buffer (*APPENDIX 2A*)in the pH range of 4.0 to 6.5 is usually satisfactory for the assay of most DPOs. Recipes for a wide range of buffer solutions may be found in *APPENDIX 2A* or in any standard textbook of practical biochemistry (for example, Dawson et al., 1986).

COMMENTARY

Background Information

Assay of diphenol oxidases

It is of key importance for any study of the kinetic properties of an enzyme that the assay procedure should measure the initial rate of the reaction; for enzymes such as DPOs that suffer product inactivation, this may pose difficult problems (Mayer et al., 1966).

The simplest, but least accurate, method of assaying DPO activity is to record the final color yield when the enzyme is incubated with a suitable chromogenic substrate such as catechol, DOPA, or 4-methylcatechol. DOPA is the most frequently used substrate in colorimetric assays because it yields a dark brown/black end-product. In this reaction, catecholase catalyzes the conversion of DOPA to dopaquinone and then to the red dopachrome, which subsequently polymerizes to yield dark brown melanin-type pigments. Unfortunately, this simple procedure has serious limitations, as it measures the end-product of a sequence of reactions rather than the true initial reaction rate. Furthermore, because different substrates yield different final colors, valid kinetic comparisons between substrates are not possible. Nevertheless, this simple assay technique has proved adequate for useful comparative studies of the levels of enzymic browning in different fruit varieties and similar problems (Vámos-Vigyázó, 1981; Machiex et al., 1990).

In order to overcome the above limitations, several workers developed a chronometric assay that involves measuring the rate of loss of ascorbate in an *o*-DPO/phenolic substrate/ascorbate coupled system; however, this is a cumbersome procedure. Other coupled assay systems have also been developed, some using quinone complexing agents such as Besthorn's hydrazone (Pifferi and Baldassari, 1973; Espin et al., 1995).

Nowadays, the most reliable method of DPO assay is measurement of the rate of O_2 uptake using an O_2 electrode. The relative merits of the different assay procedures for DPOs has been critically assessed by Mayer et al. (1966). It is now generally agreed that the polarographic O_2 electrode is the method of choice, because it has a rapid response and may be coupled to a potentiometric recorder or data logger for the immediate display of results. This method has been used routinely by many workers in biochemical studies of DPOs from fruits (Janovitz-Klapp et al., 1989; Dijkstra and Walker, 1991), cereals (McCallum and Walker, 1990), and fungi.

The O_2 electrode comprises a platinum cathode and a silver anode in an electrolyte of half-saturated KCl. When a polarizing voltage (~0.6 V) is applied the following reactions occur:

at anode:	$4Ag + 4Cl^-$	$\rightarrow$	$4AgCl + 4e^-$
at cathode:	$4e^- + 4H^+ + O_2$	$\rightarrow$	$2H_2O$
	$4Ag + 4Cl^- + 4H^+ + O_2$	$\rightarrow$	$4AgCl + 2H_2O$

Thus, current flow is proportional to the pO_2 of the solution. A simple electrical circuit converts this current to a voltage output that may then be recorded by a strip chart recorder or data logging system. The former offers a simple and convenient method of plotting the change of output voltage against time and is easy to interpret. See Clark (1992) for a more detailed description of the use the O_2 electrode.

Natural substrates

The most common naturally occurring substrates for catecholases in plants include chlorogenic acid, catechin, DOPA, and dopamine; this topic is covered in detail in the literature (see Key References). In most plants and fungi, some natural substrate(s) will be present in the tissue to be analyzed (Figure C4.1.4).

Latent DPOs

Many DPO preparations from higher plant sources, such as fruits and leaves, are particulate in nature, because, in these systems, the enzyme appears to be bound to the membranes of the chloroplast grana or to cell wall components. The O_2 electrode assay is especially advantageous for such preparations, since their turbidity could interfere with spectrophotometric assays.

These particulate, and also many apparently soluble, DPO enzyme preparations frequently exhibit the phenomenon of latency. This is manifested as atypically low DPO activity when the tissue is known to be rich in DPOs. When the enzyme is treated with a chaotropic agent such as SDS or 8 M urea, or is subjected to an acetone-precipitation procedure, a marked increase in DPO activity is revealed (Palmer, 1963; Walker and Hulme, 1965). This effect is thought to come about by the release of the membrane-bound enzyme and/or effects upon the tertiary structure of the enzyme and the active site.

Substrate specificity

The O_2 electrode assay is most useful for the comparison of substrate specificity, and the determination of K_m values, of DPOs. A major weakness of spectrophotometric assays

that measure the colored end-product of the reaction is that the color yielded by different phenolic substrates is markedly affected by the nature of the substrate. For example, DOPA gives rise to a much darker end-product than does 4-methylcatechol or chlorogenic acid (the natural DPO substrate in many fruits), yet polarographic K_m measurements show clearly that the latter is the preferred substrate.

DPO inhibitors

Similar problems may arise during investigations of DPO inhibitors. There is much interest in these, since the food industry is keenly aware of the need to control enzymic browning in many foods. Inhibition of enzymic browning can be brought about (1) by reversing or blocking the later color-producing reactions or (2) by inhibiting the actual DPO enzyme (usually a catecholase). Examples of the former include the control of enzymic browning in fruit juices by addition of ascorbic acid, which reduces the quinoid reaction products back to the parent phenol (Embs and Markakis, 1965; Golan-Goldhirsh and Whitaker, 1984), or cysteine, which combines with quinones to form colorless thioethers, thus blocking any further reaction to form colored end-products (Walker and Reddish, 1964; Montgomery, 1983; Dudley and Hotchkiss, 1989; Richard et al., 1991). By contrast, true inhibition of a DPO will involve some sort of interactive effect with the active site of the enzyme, for example, inhibition by Cu-chelating agents or cinnamic acids (Walker, 1976; Allen and Walker, 1988).

From the above discussion, it is obvious that the simple spectrophotometric DPO assays cannot be used to differentiate these two processes since they rely on measuring the colored end-products of reaction. Here, use of the O_2 electrode assay has a major advantage since it measures O_2, one of the prime reactants, and hence the actual activity of the DPO in question.

Laccases

Although catecholases are by far the most common form of DPO encountered in the food industry, laccases do occur in some fruits (Mayer and Harel, 1968; Harel et al., 1970; Joel et al., 1978; Dijkstra and Walker, 1991). However, both catecholase and laccase activity are common in many edible Basidiomycete fungi including mushrooms and shitake (Ferrar et al., 1995). Both of these enzymes use Cu as their prosthetic group. Although they carry out similar reactions, the actual reaction mechanisms are quite different. Both enzymes can oxidize a similar range of *ortho*-diphenols, but laccases can oxidize a far wider range of *ortho*- and *para*-diphenols plus other compounds. Syringaldazine is widely used as a test substrate for laccase, but it is not always oxidized by laccases from higher plants (Harvey and Walker, 1999).

Catecholases and laccases may be differentiated by the use of substrate specificity tests and selective specific inhibitors (Walker and McCallion, 1980; Ferrar and Walker, 1996; Table C4.1.1). Salicylhydroxamic acid (SHAM), PVP, and/or cinnamic acids (cinnamic, *p*-coumaric, or ferulic) are probably the best choice for catecholase inhibitors, whereas cetyltrimethylammonium bromide (CETAB) has been found to inhibit most laccases.

Critical Parameters and Troubleshooting

The preparation of extracts of plant tissue containing active DPOs can be fraught with problems. In the intact plant tissue, both enzyme and substrate are present but are thought to be compartmentalized, with the enzyme bound to membranes and the native substrate(s) present in the vacuole. As soon as the tissue is disrupted, these can react together with the very real risk of a suicidal inactivation of the enzyme by its own reaction products. As a result, most isolation procedures for DPOs include additions of ascorbate and/or cysteine to prevent the formation of the reactive quinones. Assuming that the enzyme is

Table C4.1.1 Differentiation of Catecholase and Laccase by Use of Selective Substrates and Inhibitors

	Catecholase	Laccase
Substrates:		
o-Dihydroxy phenols	Oxidized	Oxidized
p-Dihydroxy phenols	May be oxidized	Oxized
Guiacol		Oxidized
4-Hydroxyindole		Oxidized (blue)
p-Phenylenediamine	Slow or no oxidation	Oxidized
Syringaldazine		Oxidized (mauve)
Toluquinol		Oxidized
Inhibitors:		
CETAB (and other QACs)[a]		Inhibition
Cinnamic acids	Inhibition	
Fusaric acid	Inhibition	
4-Hexylresorcinol (4-HR)	Inhibition	
N-Hydroxyglycine		Inhibition
Polyvinylpolypyrrolidone (PVP)	Inhibition	
SDS	Activation	
SHAM[a]	Inhibition	
Tropolone	Inhibition	

[a]CETAB, cetyltrimethylammonium bromide; SHAM, salicylhydroxamic acid.

membrane bound, this allows subsequent separation and washing of the particulate fraction to remove the natural substrates. An alternate approach is to include an acetone precipitation step, which is most effective in removing the natural substrates and, as a bonus, usually removes any risk of latency effects.

A typical enzyme extraction procedure for soft plant issue might involve the following steps. The tissue is homogenized in a prechilled blender in ice-cold citrate/phosphate (McIlvaine's) buffer, pH 4.0, containing 0.001 M cysteine or ascorbic acid to temporarily halt enzymic browning. This is filtered through fine muslin or Miracloth to remove coarse debris (nappy liner material is a cheap and effective substitute for Miracloth). This basic extraction procedure may be enough for simple comparative assays with some plant materials. However, the presence of the natural substrate(s) in such a crude extract could lead to high levels of endogenous activity in the controls (i.e., some reaction without added substrate).

For a cleaner preparation, and to remove any residual phenolic substrates, the filtrate can be centrifuged at high speed (20,000 × *g*) at 4°C for 5 to 20 min. The supernatant is then decanted and tested for DPO activity. The precipitate is washed twice by resuspending in cold 1% KCl and recentrifuging. Finally, the washed precipitate is resuspended in 1% KCl or a suitable buffer and is stored at 4°C or deep-frozen.

For more detailed extraction procedures, refer to articles in Literature Cited.

In all types of assay, the use of enzyme extracts that are too strong or too dilute can lead to poor results. Thus, it is essential to conduct a preliminary range-finding experiment for each enzyme and each assay procedure.

It should also be noted that peroxidases and mixed-function oxidases can also bring about the oxidation of phenolic substrates; this can be checked by appropriate controls (e.g., addition of H_2O_2 to the assay system will stimulate peroxidase activity).

Polarographic O_2 electrode assays

Most problems with O_2 electrode assays can be traced back to dirty or leaking membranes; the membrane and KCl electrolyte should be replaced and the assay should be repeated. A small piece of single-thickness facial tissue between the cathode and the membrane will ensure that there is always a thin film of electrolyte present. The Pt cathode may be cleaned by gently polishing with paper tissue. Inadequate stirring and poor temperature control will also lead to erratic results.

Spectrophotometric assays

Problems that may arise with spectrophotometric assays may be due to turbid samples, inadequate mixing, or poor design of controls/blanks. Turbid samples should be clarified by centrifugation (a simple benchtop centrifuge at ~5000 × *g* should be adequate) before reading the absorbance of the clear supernatant.

Substrates

Phenolic compounds are very prone to autooxidation, so all substrates should be prepared fresh and discarded if they show signs of discoloration.

Anticipated Results

In most studies of DPO activity, the main objective is usually a simple comparison of the potential of a particular tissue to undergo enzyme-catalyzed browning, for example, a comparison of the potential for enzymic browning of different apple or mushroom cultivars. Related to this are comparative studies of different inhibitors and processing regimes to control enzymic browning. In these circumstances, it is usually sufficient to provide comparative measurements rather than absolute values of enzyme activity, in which case results can be expressed in arbitrary units such as ΔmV/min for O_2 electrode assays or Δ*A*/min for spectrophotometric assays. If more precise units are required, the O_2 electrode results should be expressed as Δnmol O_2/min/μg protein.

Time Considerations

The preparation of the enzyme extracts is usually the most time-consuming part of DPO assays and depends very much on the type of sample. If several centrifugation steps are required, it may take up to 1 to 2 hr per batch of samples.

Once the equipment is set up and equilibrated, assays with the O_2 electrode can usually be completed within 2 to 4 min per assay. Spectrophotometric assays may take ~30+ min to set up and run, but it is normal to include ~6 samples per run.

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University of Canterbury
Christchurch, New Zealand

Analysis of Lipoxygenase Activity and Products

Lipoxygenase (LOX), a non-heme iron protein, catalyzes the dioxygenation of methylene-interrupted (*Z*,*Z*)-pentadiene fatty acids to furnish conjugated hydroperoxydiene fatty acids. In the plant kingdom, the substrate fatty acids are usually linoleic acid or linolenic acids. However, plant LOXs, like their animal counterparts, are fully capable of oxidizing animal polyunsaturated fatty acids such as arachidonic acid. Most LOXs can oxidize glyceride polyunsaturated fatty acids as well. Linoleic acid usually serves as the model substrate. Hydroperoxide products of linoleic acid are usually either 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HPODE), 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9*S*-HPODE), or both (Figure C4.2.1). Other isomers have been found as LOX products, but usually the minor amounts of (*E*,*E*)-diene isomers observed are racemic, indicating that they arise from the rearrangement of fatty acid peroxyl radicals formed by either enzymic or autooxidation processes.

Although LOX activity is important to the plant's defense against pathogens, there are negative aspects of the enzyme in foods. LOX activity and the resulting fatty acid hydroperoxide products initiate free radical chains that modify proteins (particularly residues of Trp, His, Cys, Tyr, Met, and Lys) as well as vitamins or their precursors (e.g., carotene and tocopherol). Evidence of such free radical reactions is often visibly observed as loss of carotenoid/chlorophyll pigments in improperly blanched frozen foods. Another consequence of these free radical reactions is the development of potent off-flavors, many of which originate from decomposition of the fatty acid hydroperoxide products.

As seen in Figure C4.2.1, there are two obvious methods of readily measuring LOX activity, that is, by oxygen uptake and ultraviolet (UV) absorbance of the conjugated hydroperoxy-diene product. Basic Protocol 1 describes the polarographic measurement

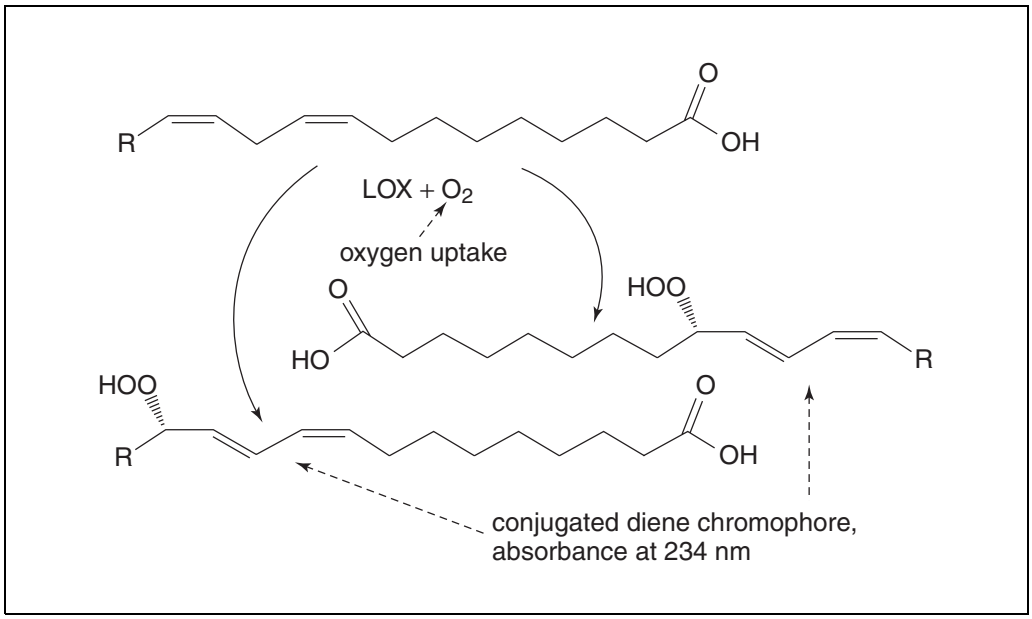

Figure C4.2.1 Lipoxygenase (LOX)–catalyzed transformation of linoleic or linolenic acid (R = $CH_3(CH_2)_4$–, linoleic acid; R = $CH_3CH_2CH{=}CHCH_2$–, linolenic acid) showing oxidation by molecular oxygen and formation of conjugated diene hydroperoxides. These events give the basis for measurement of activity by either oxygen uptake or UV absorption at 234 nm. Also shown is the usual preference for (*S*)-stereospecificity of oxidation.

Contributed by Harold W. Gardner

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of oxygen uptake by a Clark-type oxygen electrode. The oxygen electrode method is more suitable for use in the presence of other enzymes that utilize the hydroperoxide products of LOX (that is, the initial oxygen uptake is unaffected by subsequent modifications of hydroperoxide fatty acids by other enzymes). Also, the optical clarity of the reaction solution can be largely ignored using the oxygen electrode procedure. However, in some applications the stirring required with oxygen electrode measurement may be a problem. Glickman and Klinman (1996) postulated that stirring may have been the cause of a three-fold-larger K_m value determined by the oxygen electrode method compared with the spectrometric assay (Alternate Protocol 1). However, the V_{max} values for both methods were the same. Although Glickman and Klinman (1996) had no clear explanation for the discrepancy in K_m, they thought that the sheer force from stirring may have disrupted the preferred substrate, premicellar aggregates of the fatty acid.

The Alternate Protocol determines product formation by spectrophotometric detection of conjugated diene at 234 nm. The widespread availability of UV spectrophotometers may account for the use of this method by the majority of investigators. However, fatty acid hydroperoxide-utilizing enzymes must be absent, and optical clarity is a must. Thus, the spectrophotometric protocol works best with highly purified LOX preparations at high pH values, where linoleic acid exists as a more soluble anionic salt. Because of the importance of substrate solubility, LOXs having high pH optima (≥8.5), such as soybean LOX-1, are defined as type 1. LOXs with neutral or acidic pH optima, such as soybean LOX-2 and -3, are referred to as type 2.

In contrast to Basic Protocol 1 and the Alternate Protocol, which involve activity assays, Basic Protocol 2 describes procedures to determine the regio- and stereoconfigurations of product hydroperoxides by straight-phase (SP) and chiral-phase (CP) high-performance liquid chromatography (HPLC). As product composition is an important characteristic of LOXs, this measurement is included in many investigations. Support Protocol 1 describes the preparation of LOX extracts from dry material and from samples with high moisture content. Support Protocol 2 details the production of diazomethane which is required for methylating compounds prior to HPLC analysis.

BASIC PROTOCOL 1

POLAROGRAPHIC MEASUREMENT OF OXYGEN UPTAKE

Oxygen electrode equipment is used to determine oxygen uptake as a function of time. After preparing the enzyme (see Support Protocol) and calibrating the instrument, substrate and enzyme are added sequentially to a buffered solution, and the rate of oxygen uptake is monitored by a recorder.

Materials

Sodium dithionite
0.1 M assay buffer, e.g.:
  Na/K acetate, pH 4.5 to 5.5
  Na/K 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5 to 6.7
  Na/K piperazine-*N,N′*-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 6.1 to 7.5
  Na/K *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), pH 6.8 to 8.2
  Na/K borate, pH 9 to 11
  Tris·Cl, pH 7 to 9 (*APPENDIX 2A*)
LOX supernatant solution (enzyme; see Support Protocol 1)
50 mM linoleic acid substrate solution (see recipe): O_2SUBS-T1 (for type 1 LOX) or O_2SUBS-T2 (for type 2 LOX)

Oxygen monitor (e.g., YSI) equipped with constant-temperature circulating bath (±0.02°C), magnetic stirrer, recorder, Clark-type oxygen-electrode probe, 2- to 4-ml water-jacketed sample chamber, oxygen probe membranes, oxygen probe solution (half-saturated KCl), and O rings for affixing membrane

Plastic squeeze bottle

Hamilton syringes or equivalent

Additional reagents and equipment for determination of protein concentration (*UNIT B1.1*)

Calibrate electrode

1. Set up an oxygen monitor so that the circulating water bath and water-jacketed sample chamber are at 25°C.

2. Hold the electrode straight up and place a drop of KCl solution on the probe. Carefully place the membrane over the probe so that an air bubble is not trapped in the KCl solution. To hold the membrane in place, push an O ring over the end of the probe.

   Optimal oxygen detection by the Clark-type electrode is assured by a daily replacement of the membrane over the electrode. This can be very easily done with practice.

3. Place the electrode in the sample chamber and set the voltage of the electrode at –0.8 V direct current (or follow the specific instructions of the manufacturer).

4. Place a squeeze bottle half-full of water to 25°C in the constant temperature bath. Vigorously shake the bottle to equilibrate the dissolved O_2 at 25°C.

   The instrument must be calibrated to obtain a 100% response for the oxygen content of 25°C water shaken vigorously before use. Buffer solutions are saturated at slightly different levels of oxygen. Although the difference in dissolved oxygen between buffer and water is minor, it is best to use 25°C water as a known standard.

5. Turn on the magnetic stirrer and fill the chamber with the 25°C water, including the narrow bore through which the LOX and substrate solutions are injected.

   The unstirred bore serves as a static liquid column that guards against oxygen exchange.

6. Adjust the sensitivity of the instrument to nearly full span on the recorder (usually arbitrarily set at 100).

7. To zero the instrument (zero O_2 content), add ~10 mg sodium dithionite and then adjust the zero knob on the oxygen monitor instrument until zero is attained on the recorder.

   Several methods can be used to zero the instrument, such as bubbling N_2 gas into the cell. Addition of sodium dithionite is a facile way to obtain zero. Dithionite reacts with, and thus rids the solution of, dissolved oxygen.

8. Remove sodium dithionite solution using several water washes. Allow the last wash to sit a few minutes, remove the wash, and add water equilibrated at 25°C to recheck the full span level.

   The 0 to full span on the recorder using 25°C water is equivalent to 0.242 µmol O_2/ml. In a hypothetical 3-ml sample chamber, this would amount to 0.726 µmol. The instrument is now ready to make measurements.

Perform assay

9. Turn on the magnetic stirrer and fill the sample chamber with 0.1 M assay buffer equilibrated at 25°C (vigorously shake in the presence of air before using).

   It is suggested that a pH 7.5 buffer (such as Tris·Cl or HEPES) is initially used.

10. Use a Hamilton syringe to inject 1 to 50 µl LOX supernatant solution, making sure the tip of the syringe reaches the main part of the sample chamber.

    Usually 1 to 50 µl gives more than adequate response using a 3-ml chamber. This may require adjustment, including dilution of the LOX solution with buffer to obtain a reasonable measurable rate.

11. Inject 50 mM linoleic acid substrate solution.

    The order of addition of substrate and enzyme is optional.

    Injection of 6 µl containing methanolic 50 mM linoleic acid in a 3-ml sample chamber gives 0.1 mM linoleic acid as final concentration.

    Usually, there is a small oxygen burst due to greater oxygen solubility in methanol, followed by a short lag in oxygen uptake. Depending on the condition of the substrate and the amount of enzyme, a lag may not be observed. Next, a rapid linear portion is observed, usually followed by an eventual decrease as oxygen and substrate are depleted.

12. Perform a control assay by filling the cell with 25°C buffer and injecting an identical amount of enzyme but no substrate. Subtract the rate of the control from that of the assay containing substrate.

Determine activity

13. Select the most rapid portion of oxygen uptake following the short lag to draw a best-fit straight line for determination of activity (Figure C4.2.2). If desired, determine protein concentration (*UNIT B1.1*) of enzyme solution to express activity per mg protein.

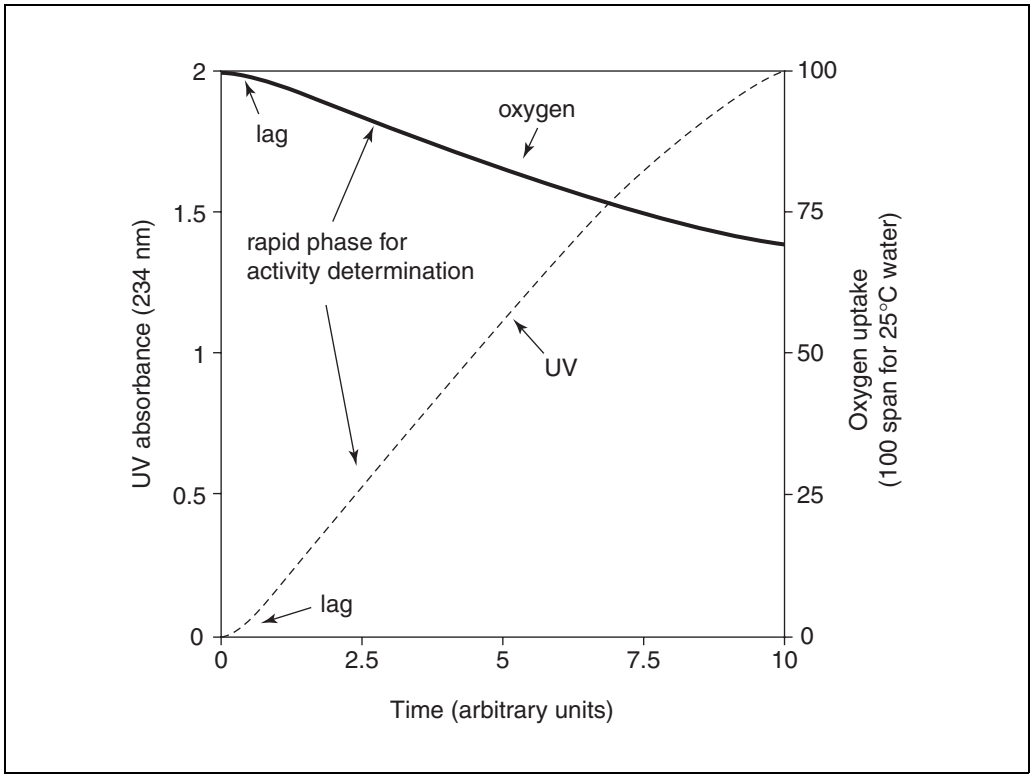

Figure C4.2.2 LOX activity measurements by both oxygen electrode (right) and UV absorbance (left). The graph shows a hypothetical reaction using identical conditions of equal substrate and LOX concentrations and temperature set at 25°C. Over the range generally used for measurement, the UV method gives a 3.24-fold greater response compared to the oxygen electrode; that is, the full-scale of oxygen uptake (0 to 100) represents 0.242 µmol O_2/ml of solution and the full-scale of UV-absorbance (0 to 2.0) represents 0.0746 µmol product/ml of solution. Also shown is the lag phase followed by a rapid linear rate used for activity calculation.

For example, on a full-scale of 0 to 100 (representing a total of 0.242 µmol O_2/ml in a hypothetical 3-ml sample chamber), rate of 10 units/min may be obtained from 10 µl LOX solution. This corresponds to a a rate of 0.0242 × 3 µmol O_2/min/10 µl or 0.00726 µmol O_2/min/µl. Activity is reported as µmol O_2/min/amount of enzyme. The amount of enzyme can be the volume of enzyme in µl or ml, or preferably the amount of protein in mg. Thus, if the activity is 7.26 µmol/min/ml and the enzyme solution has 10 mg protein/ml, then the specific activity is 0.726 µmol/min/mg protein.

At the extremes of activity rates, the measured activity may not be accurate; therefore, one should measure varying amounts of enzyme and plot the quantity of enzyme added against activity. The linear portion of the curve should determine the range of LOX volumes to be used.

14. To properly assay the LOX of interest, determine the pH optimum by assaying at pHs ranging from 5 to 11 in unit increments. For further refinement, use half units.

Some overlap with different buffers is desirable. The following buffers (all at 0.1 M) can be considered: sodium or potassium acetate (pH 4.5 to 5.5), MES (pH 5.5 to 6.7), PIPES (pH 6.1 to 7.5), HEPES (pH 6.8 to 8.2), a borate (pH 9 to 11), and Tris·Cl (pH 7 to 9).

SPECTROPHOTOMETRIC MEASUREMENT OF CONJUGATED DIENES

ALTERNATE PROTOCOL

A recording double-beam UV spectrophotometer is used to determine the conjugated diene absorbance of product fatty acid hydroperoxide at 234 nm as a function of time. Although this method is used by a majority of investigators, it has limitations regarding (1) the clarity of the enzyme and substrate solutions, (2) UV-absorbing substances in the solutions, and (3) the presence of hydroperoxide-metabolizing enzymes that destroy the conjugated diene moiety. The method works best with purified LOXs, especially type 1 LOXs. With type 2 LOXs, it is especially important to use low substrate concentrations solubilized with nonionic, non–UV absorbing detergents, like Tween 20. Compared to the polarographic method, the UV method is over three-fold more sensitive over the measurable span utilized by the two methods (Figure C4.2.2).

As for Basic Protocol 1, the LOX enzyme is prepared according to the Support Protocol. However, more care should be taken to reduce turbidity and other UV-absorbing materials caused by suspended lipid and pigments, especially when low activity is expected (requiring more enzyme addition). Triton X-100 is a UV absorber, and it causes more lipid and pigments to be suspended. Partial purification may be necessary. HEPES and PIPES buffers are not used in this method because of excessive absorption at 234 nm (0.5 to 0.6 absorbance for 0.1 M solutions); MES is useful with limitations (0.2 absorbance for 0.1 M solution).

Additional Materials *(also see Basic Protocol 1)*

Linoleic acid solution (UVSUBS; see recipe)
Double-beam UV spectrophotometer with continuous recording capability
1-cm quartz cells

1. Add 2.97 ml of 0.1 M assay buffer at 25°C to both a sample and a reference quartz cuvette.

Preferably, the cuvette holders should be temperature controlled; otherwise special attention should be given to the temperature of the reaction, such as use of reagents equilibrated at room temperature.

2. Add 30 µl linoleic acid solution (UVSUBS) to both cuvettes and stir.

Linoleic acid solution is added to the buffered reference cuvette if one anticipates turbidity caused by substrate at low pH; otherwise, LOX solution and buffer only can be added to the reference cuvette, especially if the LOX solution has turbidity or high absorbance.

3. Add an appropriate amount of LOX enzyme solution to the sample cuvette (usually 1 to 10 µl; dilution of the LOX solution may be required), stir, and immediately start UV measurement at 234 nm as a function of time.

   At the extremes of activity rates, the measured activities may not be accurate; therefore, one should measure varying amounts of enzyme and plot the quantity of enzyme added against activity. The linear portion of the curve should determine the range of LOX solution to be used.

   Optional: Certain LOXs are more peroxidative in character, as assessed by the amount of conjugated oxodiene compounds produced. These products can be assessed by monitoring at 280 nm (ε = 22,000) and comparing this rate with that obtained at 234 nm.

4. Use the most rapid activity portion after the short lag to determine activity (usually a straight line; see Figure C4.2.2). To determine the amount of hydroperoxide produced, use a molar extinction coefficient of 26,800 $cm^{-1}mol^{-1}$ liter and the equation:

   A_{234} (AU/min) = ε ($cm^{-1}mol^{-1}$ liter) × *c* (mol/liter) × cell length (cm)

   For a 3-ml sample (do not forget to include the amount of enzyme added), a 1-cm path length, and an activity of 1.0 AU/min, hydroperoxide produced = [1.0/min × 3.0 ml] / [26,800 $cm^{-1}mol^{-1}$ liter × 1 cm] = 0.112 µmol/min. If a 10-µl volume of LOX initiated the reaction, the activity would be 0.0112 µmol/min/µl LOX. Preferably, specific activity would be calculated on the basis of protein (see Basic Protocol 1, step 13).

   The molar extinction coefficient used here is higher than most literature values (23,000-25,000). The reasons for using the higher value are discussed in a review by Gardner (1997).

5. Determine pH optimum as described (see Basic Protocol 1, step 14), but do not use HEPES or PIPES buffers.

   The usefulness of 0.1 M MES is questionable as this buffer gives an absorption of ~0.2 at 234 nm; MES may be more useful as a 50 mM solution. Phosphate buffer may be used if the ionic strength does not exceed 0.1 with the caveat that the buffering power may not be sufficient. More flexibility can be obtained with a cuvette with a shorter path length (see Critical Parameters).

BASIC PROTOCOL 2

HPLC ANALYSIS OF REGIO- AND STEREOCONFIGURATION OF PRODUCTS

In order to thoroughly characterize the LOX of interest, an optional procedure is offered to determine the regio- and stereospecificity of oxidation. For this purpose a reaction volume is used that is somewhat larger than that described in Basic Protocol 1 and the Alternate Protocol. The product hydroperoxides are reduced to their corresponding hydroxides, purified by TLC, methyl esterified, and separated by straight-phase HPLC followed by chiral-phase HPLC. In this way a complete structural analysis is obtained.

Materials

0.1 M assay buffer, e.g.:
Na/K acetate, pH 4.5 to 5.5
Na/K 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5 to 6.7
Na/K piperazine-*N,N′*-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 6.1 to 7.5
Na/K *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), pH 6.8 to 8.2
Na/K borate, pH 9 to 11
Tris·Cl, pH 7 to 9 (*APPENDIX 2A*)
LOX supernatant solution (enzyme; see Support Protocol 1)

50 mM linoleic acid substrate solution: O_2SUBS-T1 (for type 1 LOX) or O_2SUBS-T2 (for type 2 LOX)
Acid (e.g., oxalic acid), aqueous solution
2:1 (v/v) chloroform/methanol
Triphenylphosphine
TLC plate: 20 cm × 20 cm × 250 µm silica gel 60 F_{254} thin-layer chromatography plates (EM Science)
50:50:1 (v/v/v) hexane/diethyl ether/acetic acid
Ethyl acetate
Nitrogen
Methanol
Diazomethane in diethyl ether (see Support Protocol 2)
Hexane
99:0.85 and 97:3 (v/v) hexane/2-propanol

Short-wave UV lamp for viewing TLC plates
Glass wool
Standard high-performance liquid chromatography (HPLC) equipment with variable-wavelength UV detection
Standard straight-phase HPLC (SP-HPLC) column (e.g., 5 µm silica, 4.6 × 250 mm; e.g., Rainin)
Chiracel OB chiral-phase HPLC (CP-HPLC) column (e.g., 10 µm, 4.6 × 250 mm; J.T. Baker)

Perform assay

1. Combine the following in a 125-ml Erlenmeyer flask and stir 15 to 30 min at 25°C.

   0.1 M assay buffer to give a total volume of 30 ml
   10 to 500 µl LOX supernatant solution
   60 µl 50 mM linoleic acid substrate solution (final 0.1 mM).

   Compared to Basic Protocol 1, the reaction is scaled up 10-fold.

   Because autoxidation products of linoleic acid would confound the results, it is preferable to isolate linoleic acid by either TLC, HPLC, or silicic acid column chromatography before using it in the reaction.

   Optional: Conducting the reaction under ice-cold conditions with stirring under a stream of pure oxygen often improves regio- and stereospecificity by reducing enzymic release of substrate free radicals. Also, a control reaction can be completed without the LOX supernatant solution. These controls typically yield very small amounts of racemic products.

Extract hydroperoxide products

2. Adjust solution to pH 4 with acid.

   Acidification with 1 M oxalic acid is convenient because the pK_a of oxalic acid is 4.

3. Immediately extract the acidified solution with 90 ml of 2:1 chloroform/methanol. Collect the chloroform layer and wash with 30 ml water.

4. Add 2 mg triphenylphosphine to the chloroform layer to reduce the hydroperoxides to hydroxides.

   At this stage the extract can be stored in the freezer (up to ~1 month at −20°C or lower) or processed further.

Prepare methyl hydroxyoctadecadienoates (Me HODEs)

5. Evaporate the chloroform extract and apply the resulting concentrated solution (~200 to 300 µl) as a band across at least half of a TLC plate. Develop with 50:50:1 hexane/diethyl ether/acetic acid.

6. Visualize the UV-absorbing bands with a short-wave UV lamp ($R_f = 0.4$), scrape them from the plate (Figure C4.2.3), and transfer to a small beaker (10 to 25 ml).

7. Add ~5 ml ethyl acetate and recover by filtration using glass wool packed into a Pasteur pipet or the stem of a funnel.

   All glassware and glass wool should be rinsed previously with ethyl acetate to remove impurities.

   The 13-hydroxyoctadecadienoic acid migrates slightly further than 9-hydroxyoctadecadienoic acid; thus, two closely migrating bands may be seen. Avoid the triphenylphosphine band at the top of the plate and the triphenylphosphineoxide band migrating slightly off the origin (Figure C4.2.3).

8. Evaporate ethyl acetate extract with a stream of nitrogen and dissolve the residue in 200 µl methanol.

9. Add 2 ml freshly prepared diazomethane in diethyl ether.

   CAUTION: *Diazomethane is a very toxic gas; keep under a fume hood.*

10. If the solution retains the yellow color of diazomethane for 30 sec, evaporate the solvent with a stream of nitrogen. Otherwise, add more diazomethane until yellow

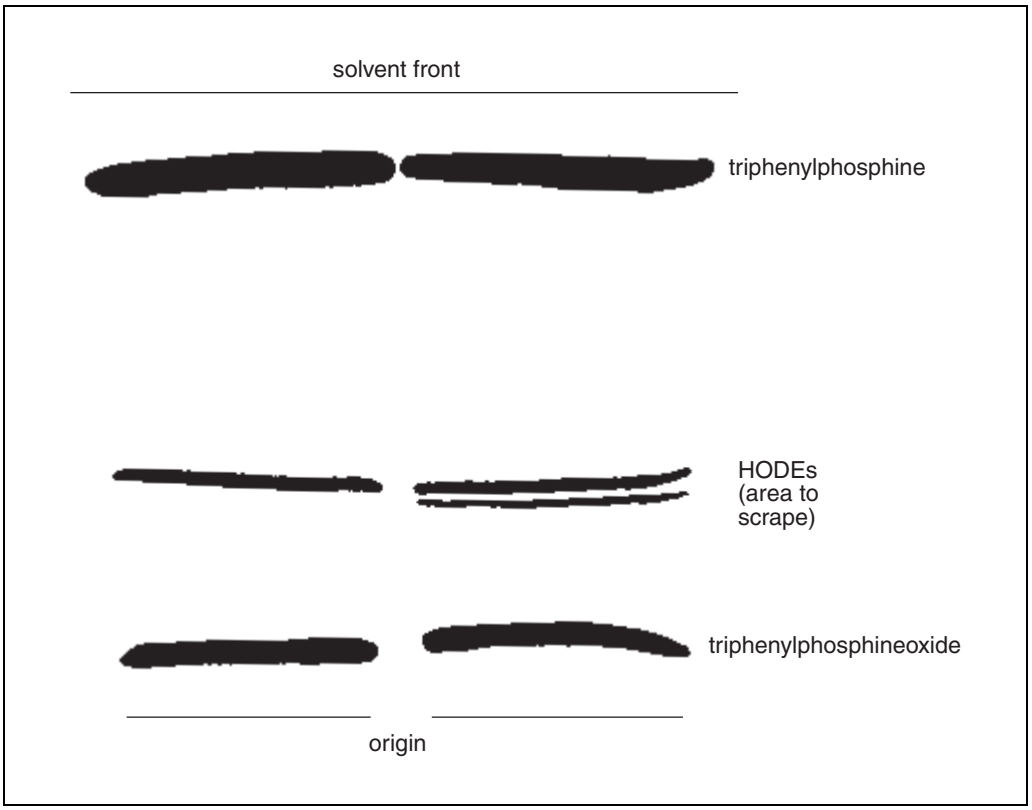

Figure C4.2.3 TLC showing separation of triphenylphosphine-reduced hydroperoxides of linoleic acid using 50:50:1 (v/v/v) hexane/diethyl ether/acetic acid. Short-wave UV visualization reveals unreacted triphenylphosphine (top), hydroxyoctadecadienoic acid (HODE) isomers (middle), and triphenylphosphineoxide (bottom). On the left, mainly 13-HODE (*E*,*Z*)-diene isomer is present; on the right, a mixture of 9- and 13-HODE, mainly (*E*,*Z*)-diene isomers, is present.

color persists for 30 sec and then evaporate the solvent including excess diazomethane.

11. Dissolve residue containing Me HODEs in 0.5 to 1 ml hexane for HPLC separation.

Separate Me HODEs by SP-HPLC

12. Inject the hexane solution of Me HODEs (usually <10 µl) onto an SP-HPLC column and elute Me HODEs with 99:0.85 hexane/2-propanol while monitoring with UV at 232 to 234 nm.

This is a modification of the method originally used by Chan and Levett (1977). The following elution order is obtained: (1) methyl 13-hydroxy-9(Z),11(E)-octadecadienoate, (2) methyl 13-hydroxy-9(E),11(E)-octadecadienoate, (3) methyl 9-hydroxy-10(E),12(Z)-octadecadienoate, and (4) methyl 9-hydroxy-10(E),12(E)-octadecadienoate (Figure C4.2.4A). Peaks 1 and 3 are collected for CP-HPLC analysis; peaks 2 and 4 are racemic

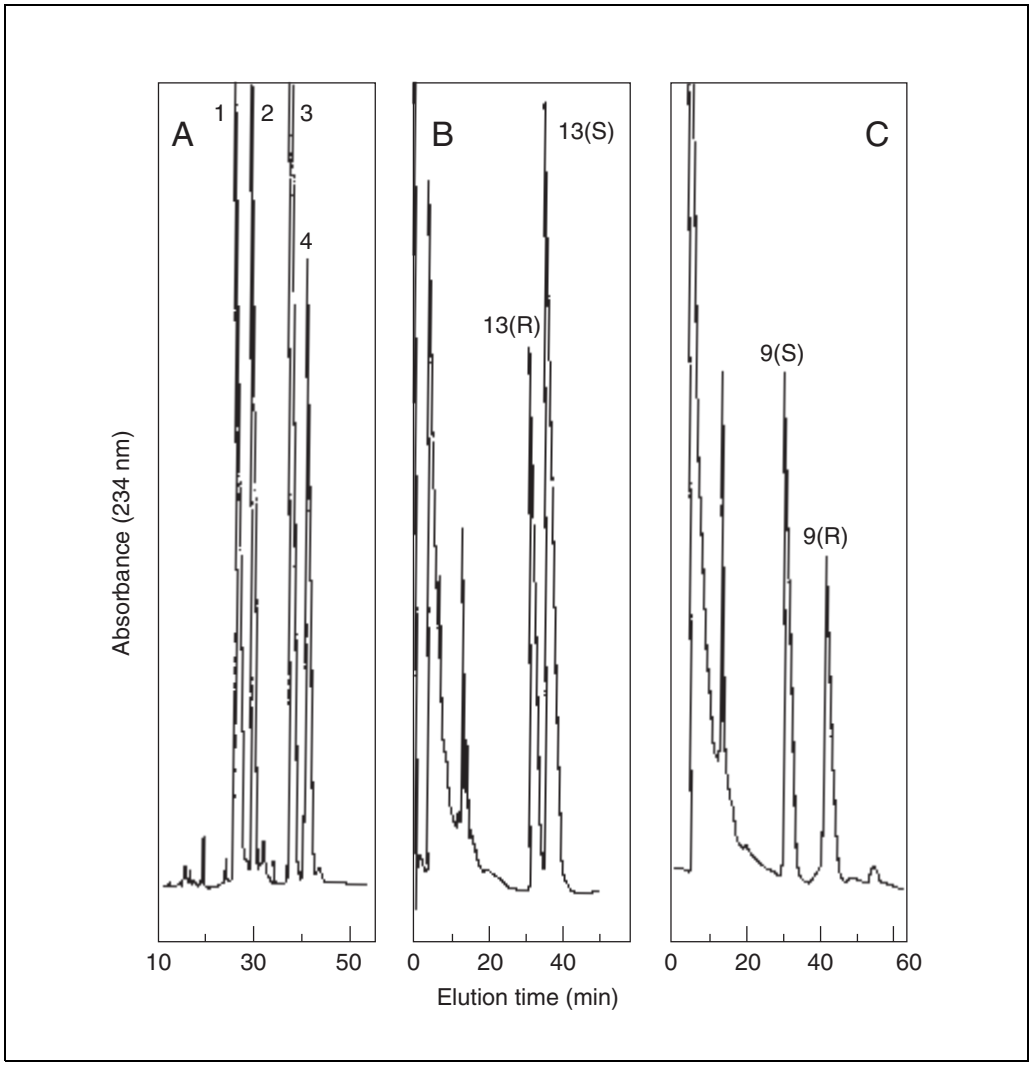

Figure C4.2.4 (**A**) SP-HPLC of methyl hydroxyoctadecadienoates obtained from linoleic acid hydroperoxide products. Peak 1, methyl 13-hydroxy-9(*Z*),11(*E*)-octadecadienoate; peak 2, methyl 13-hydroxy-9(*E*),11(*E*)-octadecadienoate; peak 3, methyl 9-hydroxy-10(*E*),12(*Z*)-octadecadienoate; peak 4, methyl 9-hydroxy-10(*E*),12(*E*)-octadecadienoate. In this chromatogram, peaks 2 and 4 are more abundant than ordinarily encountered; retention times may vary (but not the order of elution) depending on the type of silica HPLC column. (**B**) CP-HPLC of peak 1 from A. The 13(*R*)-stereoisomer elutes before the 13(*S*)-stereoisomer. Elution times may vary. (**C**) CP-HPLC of peak 3 from A. The 9(*S*)-stereoisomer elutes before the 9(*R*)-stereoisomer. Elution times may vary.

(E,E)-diene hydroperoxides and do not require CP-HPLC analysis. All four peaks are integrated to obtain the percent regiocomposition.

Preferably, the identity of Me HODEs are confirmed by gas chromatography/mass spectrometry (GC/MS) as their trimethylsiloxyl ethers (see Gardner, 1997).

Separate Me HODEs by CP-HPLC

13. Concentrate peaks 1 and 3 by solvent evaporation until a convenient volume is obtained for injection onto a CP-HPLC column. Separate peaks 1 and 3 individually using two different CP-HPLC runs to avoid overlap of peaks. Elute using 97:3 hexane/2-propanol while monitoring with UV at 232 to 234 nm.

This method is nearly identical to the method of Brash and Hawkins (1990). With methyl 13-hydroxy-9(Z),11(E)-octadecadienoate (peak 1 from step 12), the (R)-stereoisomer elutes before the (S)-stereoisomer (Figure C4.2.4B). The opposite is true of methyl 9-hydroxy-10(E),12(Z)-octadecadienoate (peak 3), where the (S)-stereoisomer elutes first (Figure C4.2.4C). Integration of the separated peaks furnishes the percent (R) and (S) composition.

SUPPORT PROTOCOL 1

PREPARATION OF LOX EXTRACTS

Methods are given to homogenize either dried tissue or fresh tissue containing a high percentage of water. Centrifugation of the homogenate gives a solution containing LOX activity. Appropriate homogenizing buffers (50 mM, pH 6 to 8) include 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N,N′*-bis(2-hydroxypropanesulfonic acid) (PIPES), *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), or Tris·Cl. One can also use 0.1 M sodium acetate, pH 4.5, with certain seeds (e.g., soybeans; see Axelrod et al., 1981). Optional amendments with polyvinylpolypyrrolidone (PVPP)/polyvinylpyrrolidone (PVP), phenylmethylsulfonyl fluoride (PMSF), sodium metabisulfite, ascorbic acid, Triton X-100/Brij 99, or EDTA may be desirable (see Critical Parameters).

Since certain LOX isoenzymes are more labile than others, the activity should be assayed as soon as practical. Freezing can partially inactivate certain isoenzymes. Thus, enzyme extracts should be stored on ice only.

Dry material (e.g., seeds)

In lieu of milling equipment, seed can be ground into flour with an inexpensive coffee grinder with occasional shaking to prevent packing of partially ground seed. With lipid-rich seed flour, repeated cold hexane or acetone extraction is used to remove oil followed by suction filtration through a Buchner funnel. The defatted seed flour is extracted with a 10-fold volume (v/w) of ice-cold 50 mM buffer. Generally, 1 g seed flour in 10 ml buffer is more than adequate. Extraction can be completed by one of the following methods on ice: (1) stirring for 1 hr, (2) grinding with a mortar and pestle, or (3) homogenizing in a blender (e.g., a Polytron homogenizer). Results may vary depending on the method used. The homogenate is filtered through four layers of cheesecloth and then centrifuged 20 min at 30,000 × *g*, 4°C. A residual floating fat layer is aspirated off using ordinary Teflon or tygon tubing attached to a vacuum flask, and the LOX-containing supernatant is collected. For alternatives see Critical Parameters.

High-moisture tissue (e.g., fruit and leaf tissue)

Tissue is homogenized with 50 mM buffer (pH 6 to 8) as outlined above for seeds. Depending on the water content and acidity of the tissue, or the LOX activity, the amount of buffer used varies from 1:1 to 8:1 buffer volume/tissue weight. For convenience, the total volume should be ≥10 ml, but smaller volumes are easily accomodated with

micro-equipment. Soft tissue, like certain fruit, is homogenized with buffer using a Waring blender. More intractable tissue, such as seedlings and leaves, should be cut into smaller pieces and then homogenized with buffer using a Polytron homogenizer or its equivalent (see above seed section). Alternatively, the tissue can be ground in liquid nitrogen with a mortar and pestle, and subsequently buffer-extracted with milder methods. Milder methods include stirring with buffer for 15 to 30 min, or homogenizing with buffer in a Ten Broek/Potter homogenizer or mortar and pestle. The homogenate is filtered through four layers of cheesecloth and then centrifuged 20 min at 30,000 × *g*, 4°C. If present, the floating fat layer is aspirated off, and then the supernatant containing LOX is collected. With fresh plant material, several buffer amendments should be considered to preserve activity (see Critical Parameters).

PREPARATION OF DIAZOMETHANE IN DIETHYL ETHER

SUPPORT PROTOCOL 2

A solution of diazomethane in diethyl ether is required to prepare the Me HODEs in Basic Protocol 2. To prepare this reagent, a nitrogen stream is saturated with diethyl ether, and this is bubbled into a mixture of Diazald, potassium hydroxide, methanol, and diethyl ether. The reaction produces a gaseous mixture of diazomethane, diethyl ether, and nitrogen. The gaseous mixture can be used immediately or trapped and stored in cold diethyl ether. This method is from Gardner (1997) and Schlenk and Gellerman (1960). For another description of the required apparatus, refer to the latter reference.

Materials

Nitrogen stream
Diethyl ether, room temperature and cooled with dry ice
1:2:2 (v/v/v) KOH/methanol/diethyl ether, made using 40% (w/v) aqueous KOH
N-Methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald; Aldrich)

Gas-tight vessels:
125-ml Erlenmeyer flasks equipped with double-bore rubber stoppers
Storage vessels (e.g., Teflon-lined screw-cap test tubes), optional
Tubing: Tygon and Teflon

Generate diazomethane

1. Set up two gas-tight vessels (e.g., 125-ml Erlenmeyer flasks) so that a stream of nitrogen gas can be passed through them in sequence as follows: use Tygon tubing to connect the nitrogen source to the first vessel and then to connect the first to the second vessel. However, use small-bore Teflon tubing to connect the second vessel to either the sample or a storage vessel.

   Teflon tubing provides flexibility and does not contain plasticizers to contaminate the final diazomethane/diethyl ether solution.

2. Fill the first vessel approximately half way with diethyl ether and bubble the nitrogen through the ether so that it becomes saturated with ether.

   Use a tapered tube (e.g., Pasteur pipet) to introduce the nitrogen so fine bubbles will be formed in the ether.

3. Add 50 ml of 1:2:2 KOH/methanol/diethyl ether to the second vessel. Bubble the diethyl ether–saturated nitrogen from the headspace of the first vessel into this solution.

   As described above, use a tapered tube to introduce the gas so fine bubbles will be formed in the solution. The bubbles will serve to sweep the diazomethane out of solution.

4. Start diazomethane generation by adding several grams of Diazald to the second vessel.

   Gaseous diazomethane, diethyl ether, and nitrogen collect in the head space of the second vessel. This gaseous solution can be trapped in dry ice–cooled diethyl ether for storage and later use (steps 5 to 7) or can be used immediately by bubbling directly into a methanolic sample.

Store diazomethane/diethyl ether (optional)

5. Pass the gaseous diazomethane and diethyl ether into a pretested gas-tight storage vessel (e.g., Teflon-lined screw-cap test tube) containing dry ice–cooled diethyl ether.

6. Store tightly sealed for up to a few months at −20°C.

   The yellow color of diazomethane is an indicator of the potency of the reagent after storage.

7. To use, transfer diethyl ether containing diazomethane to a sample that has been dissolved in a small amount of methanol.

   The presence of ~10% methanol in the sample is essential to increase the reaction rate of diazomethane.

REAGENTS AND SOLUTIONS

Use distilled or distilled and deionized water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Linoleic acid substrate solution, 50 mM

O_2SUBS-T1: Dissolve 28 mg linoleic acid in 1.97 ml methanol (total volume 2 ml). Seal in a 2-ml vial with a screw cap equipped with a Teflon-lined septum, and store up to a few months under argon at −20°C.

A small amount of hydroperoxides arising from autoxidation is not a major concern as it shortens the observed lag phase. Because of the insidious nature of autoxidation, the condition of the linoleic acid sample used to prepare the solution should be known. It is recommended that a fresh sealed vial from the supplier is used.

O_2SUBS-T2: Prepare as for O_2SUBS-T1, but add 28 mg Tween 20 and decrease methanol to 1.94 ml (total 2 ml).

Linoleic acid solution (UVSUBS), 10 mM

Add 28 mg linoleic acid and 28 mg Tween 20 to a small volume of water (~3 to 5 ml). Thoroughly emulsify by shaking, vortexing, or sonicating. Add 1 N KOH or 1 N NaOH drop-wise until the solution just clarifies. Adjust to pH 9.0, and bring to 10 ml with water. Divide into portions and store no more than a few weeks at −20°C under argon.

Aqueous solutions of linoleic acid tend to autoxidize more readily.

COMMENTARY

Background Information

Not all LOXs are identical in their mode of action. For example, certain LOXs are more peroxidative in character, such as soybean LOX-3 (Axelrod et al., 1981). With these peroxidative LOXs, peroxyl and/or alkoxyl radicals are thought to cause cooxidation reactions and catalyze the formation of oxodienes. Oxodiene fatty acids are detected by absorbance at 280 nm. However, most LOXs form oxodienes under partial anaerobic or oxygen-starved conditions (Garssen et al., 1971). 13-Oxo-9,11-octadecadienoic acid, 13-oxo-9,11-tridecadienoic acid, and 2,4-decadienal have been observed. This occurs because the lack of oxygen disrupts the iron redox cycle of LOX (Gardner, 1991).

Another aspect of LOX regards its specificity of product formation. The peroxidative-type LOX mentioned above tends to form a signifi-

cant amount of hydroperoxides with (*E*,*E*)-diene content. Hydroperoxides possessing (*E*,*E*)-diene conjugation are known to be formed through peroxyl radical rearrangement (Porter and Wujek, 1984), and thus, in the author's experience, the hydroperoxide group is found not only to be largely racemic but also an equal mixture of 9- and 13-hydroperoxides. With nonperoxidative types of LOX under sufficient oxygen tension, (*E*,*Z*)-diene hydroperoxides predominate, and usually plant LOXs afford mainly the (*S*)-stereoisomer of both 9- and 13-hydroperoxides. With this type of LOX there is often a preference for regioisomers, e.g., either 13*S*-HPODE, 9*S*-HPODE, or a mixture of both. In the LOX-catalyzed formation of 13*S*-HPODE and 9*S*-HPODE, the comparative H removal and O_2 insertion is opposite, leading investigators to believe that an opposite orientation of the substrate occurs within the LOX active site. Opposite orientation would give spatial identity (Lehmann, 1994). As a consequence, it is thought that high-pH type 1 LOXs give mainly 13*S*-HPODE. This occurs because the linoleate anion is repelled from the hydrophobic pocket of the active site, but the ω end does fit. At lower pHs, type 1 LOX does afford a percentage of 9*S*-HPODE, indicating that the nonionic carboxylic acid function does fit into the hydrophobic site (Gardner, 1989). Along these lines, type 2 LOXs with neutral or acidic pH optima usually yield either 9*S*-HPODE or a mixture of 9*S*-HPODE and 13*S*-HPODE. Since amino acid substitution in the hydrophobic region of LOX has been shown to cause changes in regiospecificity (Sloane, 1996), the pH of incubation is not the only factor involved.

The observed lag phase of activity, seen in Figure C4.2.2, is variable in duration and may not be noticed. The lag is generally thought to be due to the time required to convert inactive native Fe^{2+}-LOX into active Fe^{3+}-LOX. Thus, some amount of fatty acid hydroperoxide product is required to "prime the pump." As a consequence, relatively long lag phases are often due to either low LOX concentrations, highly purified substrates containing no hydroperoxides from autoxidation, or both.

Critical Parameters

There are alternatives to consider when extracting LOX from dry seed material. Instead of oil removal by hexane/acetone extraction, oil can be partially removed by vacuum aspiration of the fat pellet floating on the supernatant after centrifugation. As an alternative to extraction with neutral buffers, the more acidic 0.1 M sodium acetate (pH 4.5) is often used to exclude extraction of unwanted protein, such as with soybean flour (Axelrod et al., 1981), but the pH must be readjusted to 6.8 after centrifugation. For further purification of LOX, the literature should be consulted. Typical methods include $(NH_4)_2SO_4$ precipitation (between 30% and 60% of saturation) and chromatography on DEAE-type supports (e.g., Axelrod et al., 1981).

In the preparation of LOX enzymes from fresh plant tissue, the following amendments to the homogenizing buffer should be considered in order to ameliorate factors detrimental to activity. With tissue containing phenol oxidases, like potato tuber (enzymic browning), detrimental polyphenolic-protein complexes can be largely avoided by amending the buffer with one or more of the following: 1% to 5% PVPP or PVP, 2 mM sodium metabisulfite, and/or 1% ascorbic acid. In tissue suspected to be high in protease activity, such as certain seedlings, add 1 mM PMSF (*NOTE:* PMSF is highly toxic; avoid skin contact). Since PMSF hydrolyzes rapidly, it should be added directly to the newly prepared homogenate (dissolved in a small volume of acetone); PMSF should not be predissolved in the buffer. Nonionic detergents, like 0.1% Brij 99 or 0.1% Triton X-100, have been reported to improve extraction of LOX. Some investigators include 1 mM EDTA to scavenge free metal ions.

Prior to assay, all LOX enzyme solutions should be kept ice-cold through all steps of the preparation procedure. The literature indicates that LOX solutions can be successfully frozen and stored after addition of 10% to 20% glycerol. In the author's laboratory, soybean LOX-1 is stored in the refrigerator (~4°C) as a suspension in 2.3 M $(NH_4)_2SO_4$, and this procedure has proved to maintain good activity for years.

If a quantity of 1 to 50 µl LOX solution does not yield a sufficient response using the oxygen electrode assay, larger quantities may be used to replace part of the buffer. In this case, pH adjustments may be necessary. Larger quantities may require time for temperature equilibration in the sample chamber, and possibly equilibration of dissolved oxygen (a stream of air bubbled into the solution can correct this problem).

Using the UV assay, very active LOX preparations, such as soybean preparations, can tend to be confusing, because the reaction is essentially over before measurements can be taken. This may lead the novice into the belief that

there is no activity present. This error can be checked by sequential 10-fold dilutions (10-, 100-, and 1000-fold). Also, the presence of an efficient hydroperoxide-metabolizing enzyme, such as allene oxide synthase (AOS), can lead to essentially little or no actual UV absorbance changes. This author has observed the latter effect with LOX preparations from *Zea mays* embryo. Simple procedures, such as selective removal of AOS by pH adjustment (pH 4.5 and centrifugation), subsequently revealed LOX activity.

Regarding the UV assay, excessive absorption may be encountered in certain cases. For example, the Good buffers PIPES and HEPES absorb substantially at 234 nm. The optional use of a 9-mm optically transparent quartz spacer inserted into a 1-cm cuvette gives a 10-fold advantage with solutions of high UV absorbance. The spacer also serves as a convenient mixer, whereas mixing is difficult with specialized 1-mm cuvettes.

While completing sequential assays, some residual activity might be observed. There may be some adsorbed LOX on the glass, quartz, or Teflon-coated stirrer, especially with high activities. In these cases, the sample chamber or cuvette can be washed with 0.1% Triton X-100 or 75% methanol followed by water washes.

Because of the insolubility of LOX substrates, special consideration must be given to their preparation. Using an oxygen electrode, there is no concern for optical clarity; thus, injection of methanolic solutions of linoleic acid gives an initial dispersal of linoleic acid into micro-droplets. With buffers having pH values above 8.5, the micro-droplets are rapidly transformed into more soluble linoleate anions. Methanolic solutions have also been used in the UV assay to study kinetics of type 1 LOX at basic pH values (Schilstra et al., 1993). This procedure was satisfactory because the concentrations used were below the critical micelle concentration (CMC; 10 to 80 μM). At pH values below 8.5, it is advisable to include a nonionic detergent to disperse linoleic acid or other fatty acid substrates, even using the oxygen electrode method. With the UV assay, it is more critical to have optimal substrate dispersal at the outset because of the requirement for optical clarity. Most investigators use modifications of the method originally described by Surrey (1964), which utilized equal weights of linoleic acid and Tween 20. In this procedure, linoleic acid was emulsified by Tween 20 and then converted to its anion with NaOH. Finally, the substrate was adjusted to pH 9 with borate buffer.

To prepare UVSUBS, the author uses the method of Axelrod et al. (1981), which does not include borate buffer. Thus, the pH 9 UVSUBS does not greatly affect the pH when added to the more acidic 0.1 M buffers. Even with amendment by Tween 20, the final concentration of linoleic acid reportedly cannot exceed 1 mM in more acidic buffers, due to development of cloudiness. Although nonionic detergents (non-UV absorbing) have been used to improve optical clarity at lower pH values, detergents often inhibit activity by sequestering substrate within micelles (Schilstra et al., 1994). Due to substrate inhibition at high linoleic acid concentrations, small amounts of detergent actually cause increases in activity in these cases (Schilstra et al., 1994). Essentially, monomeric substrate, the actual form utilized by LOX, is released from detergent/substrate micelles by an equilibrium process. For further information on activity inhibition by increasing concentrations of Tween 20, see Srinivasulu and Rao (1993). These latter authors have also studied the effect of alcohols on LOX activity (Srinivasulu and Rao, 1995). Methanol used in the O_2SUBS-T1 and O_2SUBS-T2 is fairly minimal (0.2%), and this quantity should give >95% of the without-methanol activity. Glycerides as substrates have been dispersed by deoxycholate (Brash et al., 1987; Piazza and Nuñez, 1995). The oft-reported stimulation of activity by Ca^{2+} ions apparently is due to a change in linoleic acid micelles caused by this ion; that is, Ca^{2+} is not an authentic cofactor (Galpin and Allen, 1977). In conclusion, the inhibitory effects of detergent or methanol are less of a concern to investigators seeking relative activities among samples.

If precise kinetic parameters, such as K_M, are to be determined, more care must be taken to prepare soluble substrates in monomeric form. Solutions of linoleic acid must be below the CMC, which has been determined to be 0.17, 0.06, and 0.02 mM at pH 10, 9, and 8, respectively (Verhagen et al., 1978). Also, stirring should be avoided (Glickman and Klinman, 1996), restricting the method to the Alternate Protocol.

The use of phosphate buffer is commonly reported in the literature, probably because it is a convenient neutral pH buffer that does not absorb UV at 234 nm. However, it has been shown that LOX is sensitive to the ionic strength of buffers (Christopher et al., 1970). For example, it was determined that the opti-

mum ionic strength of phosphate buffer was up to 0.1, after which the relative activity decreased substantially as ionic strength increased (Zhang et al., 1991). For this reason, it is recommended that monovalent buffers be utilized. Using the formula for ionic strength (μ): $\mu = \frac{1}{2}\Sigma\, c_i Z_i^2$ where c_i is the concentration of the ith ion, Z_i is its charge, and the summation includes all ions in solution. It is seen that the ionic strength of 0.1 M potassium (or sodium) borate is identical to its molarity: $\mu = \frac{1}{2}[(0.1)(1)^2 + (0.1)(1)^2] = 0.1$

Anticipated Results

Figure C4.2.2 summarizes anticipated results from the use of Basic Protocol 1 (oxygen uptake method) compared with the Alternate Protocol (UV method). Figure C4.2.2 shows hypothetical responses obtained with an equal concentration of LOX and substrate by the two methods.

The procedures outlined in Basic Protocol 2 will afford a total analysis of LOX products analyzed as their methyl esterified and reduced derivatives. For example, a typical result would be reported as follows: 86% methyl 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate, 1% methyl 13(*R*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate, 2% methyl 13(*R*,*S*)-hydroxy-9(*E*),11(*E*)-octadecadienoate, 8% methyl 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoate, 1% methyl 9(*R*)-hydroxy-10(*E*),12(*Z*)-octadecadienoate, and 2% methyl 9(*R*,*S*)-hydroxy-10(*E*),12(*E*)-octadecadienoate.

Time Considerations

Accumulating the required materials (substrate(s), buffers, and amendments) may take some time; thus, they should be prepared the day prior to making measurements. For Basic Protocol 1 and the Alternate Protocol, the first time the procedures are tested, it may take the better part of a day. Familiarity with the procedures should shorten the time required to 2 to 3 hr, especially if the pH optima of the LOXs are known. With previously prepared LOX samples and reagents, 30 min is adequate.

Using Basic Protocol 2, it is suggested that the LOX reaction and preparation of the methyl hydroxyoctadecadienoates are completed one day, and the HPLC separations be done the next day.

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Extraction and Measurement of Total Lipids

Lipids are soluble in organic solvents, but sparingly soluble or insoluble in water. The existing procedures for the extraction of lipids from source material usually involve selective solvent extraction and the starting material may be subjected to drying prior to extraction. Solubility of lipids is an important criterion for their extraction from source material and depends heavily on the type of lipid present, and the proportion of nonpolar (principally triacylglycerols) and polar lipids (mainly phospholipids and glycolipids) in the sample; therefore, several solvent systems might be considered, depending on the type of sample and its components. Incomplete removal of lipids via cold pressing can be employed, such as is in practice for the extraction of oil from flaxseed or screw-pressing of seeds like canola, prior to solvent extraction. The solvents of choice are usually hexane, in the case of Soxhlet (AOAC, 1995; see Basic Protocol 1) and Goldfisch methods (see Alternate Protocol 1), chloroform/methanol or chloroform/methanol/water, in the case of the Folch Method (Folch et al., 1957; see Basic Protocol 2) or its modified Bligh and Dyer Procedure (Bligh and Dyer, 1959; see Alternate Protocol 2), and *n*-propanol/water for high-amylose containing starchy foods (see Basic Protocol 3).

In addition, to release lipids from source material, such as those in starch, fish meal, or milk, it might be necessary to treat the sample with an acid prior to lipid extraction (see Basic Protocol 4). In the case of milk, addition of ammonium hydroxide is necessary to dissolve casein prior to lipid extraction, which will release the lipids from its surrounding matrix (e.g., from the film surrounding the fat globules in milk). Furthermore, in certain cases, it is necessary to predry the sample in order to allow efficient and complete extraction of lipids. Particle size reduction is another factor that may improve lipid extraction efficacy.

Accurate determination of lipids in foods is required for nutritional labeling, certification, or for evaluation of standard of identity and uniformity, as well as examination of their effects on functional and nutritional properties of foods. Following lipid extraction and precise quantitative analysis, lipids so obtained may be used for analysis of other lipid characteristics and properties provided that nondestructive and mild extraction procedures are employed that retain the integrity of lipids. Thus, determination of lipid classes, fatty acid composition (*UNIT D1.3*), and oxidative state of lipids (Chapter D2), amongst others, may be pursued following the extraction process.

CAUTION: The solvents used in these protocols are flammable, treat accordingly. See *APPENDIX 2B* for more information.

IMPORTANT NOTE: Use only ACS grade reagents and deionized water.

NOTE: A balance accurate to 0.1 mg is recommended for all measurements.

SOLVENT EXTRACTION OF OILSEEDS, NUTMEG, AND OTHER FOODS USING THE SOXHLET METHOD

BASIC PROTOCOL 1

Extraction of lipids into hexane, low-boiling petroleum ether (a mixture of pentanes and hexanes with a boiling point of 35° to 40°C), as well as diethyl ether, is easily achievable, provided that the moisture content of the food sample does not exceed 10%. This method may be used for quantitation of lipids in both low-fat and high-fat source materials but it removes mainly nonpolar lipids from samples as polar lipids are generally scarcely soluble in nonpolar solvents. In the case of high moisture foods, predrying of the sample may be necessary. Since high temperature drying procedures may adversely affect the

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oxidative state of lipids, predrying may be achieved using low temperature drying of the sample under vacuum (<100 mm Hg) at 40° to 50°C overnight, or 95° to 100°C for 5 hr (AOAC, 1995). In some cases, such as determination of fat content in meat samples by Goldfisch method (see Alternate Protocol 1), mixing of the sample with sand is necessary to avoid crust formation, which may lead to incomplete lipid extraction. This facilitates penetration of the solvent into the food matrix, increases surface area, and may hasten size reduction, if necessary. The Soxhlet extraction is the method commonly used; however, an alternate Goldfisch extraction procedure may also be employed for this purpose (see Alternate Protocol 1).

The Soxhlet extraction procedure is a semicontinuous process, which allows the buildup of the solvent in the extraction chamber for 5 to 20 min. The solvent surrounds the sample and is then siphoned back into the boiling flask (Fig. D1.1.1). Multiextractor units are available for extraction of lipids from several different samples or replicate runs of the same material. The procedure provides a soaking effect and does not permit channeling. The fact that polar and bound lipids are not removed is a drawback to the procedure (see Background Information).

Materials

60 g canola, mustard, soybean, or nutmeg seeds
Hexane

Coffee grinder
Soxhlet apparatus:
  Cellulose extraction thimbles and glass wool
  Condenser
  500-ml flat-bottom flask, predried with boiling chips or glass beads
  Heating mantle
  Soxhlet extractor
Rotary evaporator

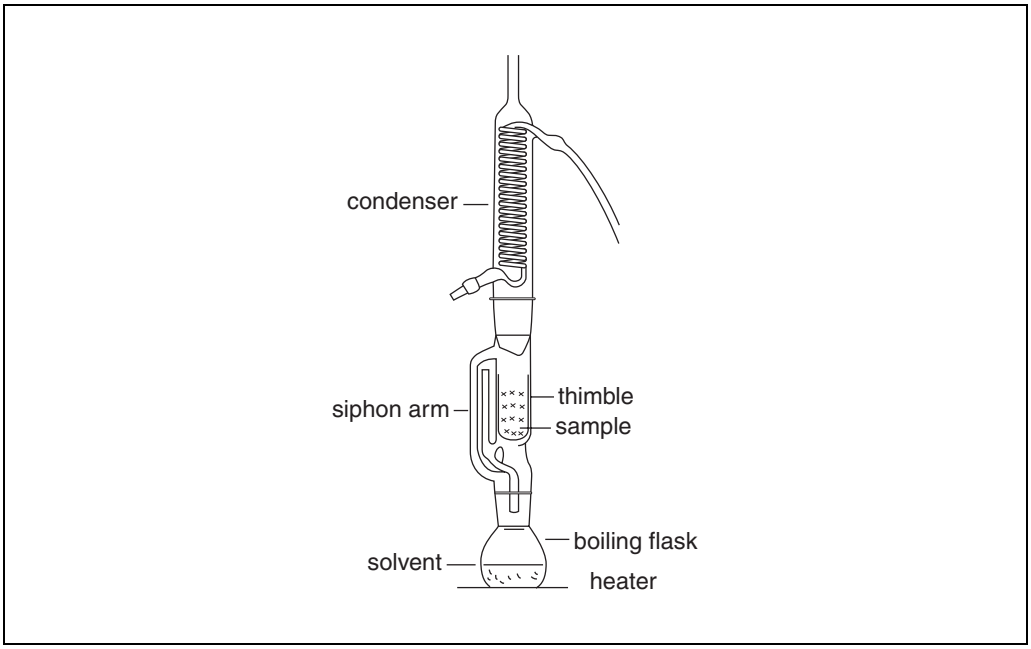

Figure D1.1.1 Soxhlet lipid extraction unit used for continuous extraction of analytes from a solid into an organic solvent. As the flask containing the solvent is heated, vapors rise in the larger outside tube, through the thimble containing the sample, enter the water-cooled condenser, and liquefy. When the liquid level in the extractor reaches the top of the siphon arm, the extract-enriched solvent returns to the flask.

1. Grind 60 g seeds or nutmeg in a coffee grinder with occasional shaking for 1 to 2 min.
2. Weigh exactly 20 æ 1 g of sample into cellulose extraction thimbles. Cover the top of each thimble with glass wool to prevent floating.
3. Weigh the predried flat-bottom extraction flask with a few boiling chips or glass beads.
4. Extract lipids with 150 to 200 ml of hexane at the boiling point for 7 to 12 h in a Soxhlet extractor (Fig. D1.1.1) using a heating mantle.

   The condensation rate for the solvent should be set at about 2 to 6 drops per second, depending on the extraction period envisaged. For longer extraction periods, a lower condensation rate is selected and vice versa. Usually an extraction period of 8 hr at a rate of 150 drops per min is considered adequate.

   The boiling point of hexane is ~69°C.
5. Let the sample cool.
6. Remove the solvent from the extract in a rotary evaporator at 40°C under reduced pressure.
7. Calculate the amount of lipid recovered and its percentage in the original sample as given below:

   Mass of lipid = (weight of the flask + boiling chips + extracted oil) − (weight of the flask + boiling chips)

   Lipid content (%) = mass of lipid extracted (g)/sample weight (g) × 100

GOLDFISCH METHOD FOR LIPID EXTRACTION

ALTERNATE PROTOCOL 1

The Goldfisch extraction procedure allows the solvent (usually hexane, petroleum ether, or diethyl ether) to continuously flow over the sample held in a ceramic thimble (Fig. D1.1.2). This procedure is again useful for determination of nonpolar lipids and is faster and more efficient than the Soxhlet extraction method (see Basic Protocol 1); however, it may cause channeling, which could render extraction of lipids incomplete. Although diethyl ether is a more efficient solvent, in general, danger of explosion, fire hazard, and a higher price are among the reasons for selection of low boiling petroleum ether (35° to 60°C) for lipid extraction.

Additional Materials *(also see Basic Protocol 1)*

10 g sample, predried
Petroleum ether

Goldfisch lipid extraction unit (e.g., Labconco Corp.):
- Extraction container, predried
- Glass holding tube
- Forced-air oven

Dessicator

CAUTION: The Goldfisch lipid extraction unit should be operated inside an explosion-proof fume hood to exhaust the vapors from flammable solvents used during normal operation. The fume hood should be equipped with a fire suppression device.

1. Weigh predried porous ceramic extraction thimbles and the predried extraction container. Add 10 g predried sample to the thimble and weigh again.
2. Place the ceramic extraction thimble into a glass holding tube.

3. Place ~40 ml of hexane or petroleum ether in the extraction container.
4. Set the equipment to extract for 4 to 7 hr.
5. Let the sample cool.
6. Remove the extraction container and then evaporate the solvent in the air overnight with subsequent heating at 95° to 100°C for 30 min using a forced-air oven.
7. Cool the beaker in a desiccator. Weigh the beaker and its contents. Calculate the weight of the lipid:

   Weight of lipid = (weight of container + extracted lipid) − (weight of container)

   Alternatively, the sample may be transferred before solvent removal to a tared round bottom-flask. Solvent may then be removed at 40°C under vacuum in a rotary evaporator.
8. Calculate the percentage of lipid in the sample as given below.

   Lipid content (%) = mass of lipid extracted (g)/sample weight (g) × 100

   Extracted lipids in this procedure may be used for certain further analysis, such as determination of fatty acid profile (UNIT D1.2) and oxidative state (UNITS D2.1 & D2.2) of the oil; however, for lipid classification, incomplete extraction of polar lipids into nonpolar solvents may not allow for accurate determination and quantitation.

BASIC PROTOCOL 2

CHLOROFORM/METHANOL/WATER EXTRACTION OF LIPIDS FROM MUSCLE FOODS

Mixtures of chloroform and methanol have had wide use as lipid extractants (e.g., Bligh and Dyer, 1959). This solvent system allows for extraction of both polar and nonpolar lipids, unlike extraction with hexane (see Basic Protocol 1 and Alternate Protocol 1). Optimum extraction may be achieved when water in the tissue, or that added to the medium, yields a monophasic solution. Subsequently, additional water and/or chloroform

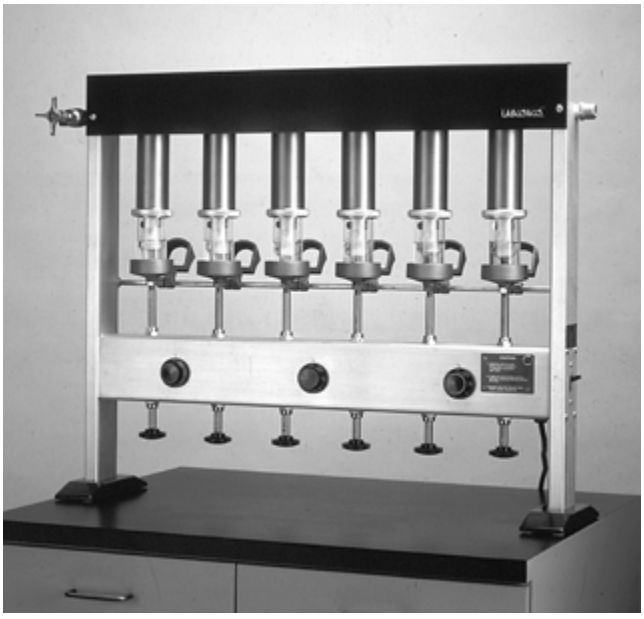

Figure D1.1.2 Goldfisch lipid extraction unit (Courtesy of Labconco Corporation, Kansas City, MO). A single beaker is used as the solvent chamber. Samples are placed between a boiling solvent and a cold surface. The solvent vaporizes, condenses on the cold surface, and washes down through the samples into the boiling solvent below.

may be introduced to produce a biphasic system. The lower chloroform layer includes the lipids and the top methanol-water layer generally contains the nonlipid components. The lipid in the chloroform layer is isolated using a separatory funnel.

CAUTION: Due to the hazardous nature of chloroform, procedures using chloroform must be conducted under a fume hood. See *APPENDIX 2B* for more information.

Materials

50 g small cut pieces of fish or meat
Methanol
Chloroform
1:1 (v/v) chloroform/methanol
Sodium sulfate, anhydrous

Waring blender or Polytron homogenizer
Tabletop centrifuge, rotor, and 50- to 500-ml tubes, or sintered glass funnel
Whatman No. 1 filter paper in a Buchner funnel
Separatory funnel
Rotary evaporator

1. Homogenize 50 g small cut pieces of fish or meat in a Waring blender, or with the aid of a Polytron homogenizer, in 100 ml of methanol. Add 50 ml chloroform. Homogenize for 2 min.

   If using a dry sample such as fish meal, use 10 to 15 g sample and add 35 to 40 ml water before homogenization.

2. Add 50 ml additional chloroform and homogenize 30 sec. Add 50 ml water and homogenize 30 sec.

3a. *For collecting liquid by centrifugation:* Transfer to 50 to 500 ml centrifuge tubes. Collect liquid in a tabletop centrifuge at 3300 × *g*, at 5° to 25°C. Decant and retain liquid.

3b. For collecting liquid by filtration: Filter through a sintered glass funnel or through Whatman No. 1 filter paper in a Buchner funnel with slight suction. Press the solids with the bottom of a beaker to ensure maximum solvent recovery. Retain liquids.

4. Repeat the procedure (step 3a or b) on the resulting solids, together with filter paper if applicable, by adding 20 ml 1:1 (v/v) chloroform/methanol. Retain liquids.

5. Combine the liquids and transfer into a separatory funnel.

   The bottom chloroform layer includes the lipids and should be retained for further work up.

6. Pass the chloroform layer through a 2.5-cm thick layer of anhydrous sodium sulfate using Whatman No. 1 filter paper in a funnel. Wash with 20 ml 1:1 (v/v) chloroform/methanol.

7. Remove the solvent using a rotary evaporator under vacuum, at 40°C. Calculate the weight of the lipid:

   Weight of lipid = (weight of container + extracted lipid) − (weight of container)

8. Determine the content of lipids in the sample by weight difference:

   Lipid content (%) = amount of lipid extracted (g)/weight of original sample (g) × 100

ALTERNATE PROTOCOL 2

CHLOROFORM/METHANOL/WATER EXTRACTION FROM SMALL SAMPLES

Extraction of lipid from small amounts (<1 g) of sample, such as that of rotifers, artemia, and larvae, may be achieved using an alternate procedure. In this approach, homogenization of sample is achieved using a vortex due to the delicate nature of tissues involved and the ease of their disintegration. The recovered lipids, similar to that in the original protocol (see Basic Protocol 2), may be used for further analysis.

Additional Materials *(also see Basic Protocol 2)*

300 to 400 mg sample to be analyzed (e.g., rotifers, artemia, larvae)
Methanol containing (optional) 500 ppm *tert*-butylhydroquinone (TBHQ)

15-ml test tube
Ultrasonic bath
Funnel and Whatman no. 1 filter paper

NOTE: In general, antioxidants (e.g., TBHQ), are required when extracting oils that contain highly unsaturated fatty acids with 3, 4, 5, or 6 double bonds.

1. Accurately weigh 300 to 400 mg of sample into a 15-ml test tube.
2. Add 4 ml methanol, containing 500 ppm *tert*-butylhydroquinone (TBHQ) as an antioxidant, if needed.
3. Add 2 ml chloroform and 0.4 ml water; homogenize (for larvae) and/or vortex for 30 sec. Sonicate in an ultrasonic bath for 15 min.
4. Add 2 ml chloroform and 2 ml water. Vortex for 30 sec.
5. Centrifuge the tubes at 3300 × *g* for 15 min, at 5° to 25°C.
6. Remove the upper layer (methanol/water) using a Pasteur pipette.

   There may be solids at the interface which should be allowed to remain in the tube.
7. Transfer the lower layer (methanol/chloroform) into a clear tube with a Pasteur pipette. Leave the solids behind, if present.
8. Reextract the solids left at the bottom of the test tube by repeating steps 4 to 7 using 2 ml of 1:1 (v/v) chloroform/methanol in step 4.
9. Pass the combined layers through a 2.5-cm thick layer of anhydrous sodium sulfate using Whatman no. 1 filter paper in a funnel, into a preweighed container suitable for a rotary evaporator.
10. Remove the solvents using a rotary evaporator under reduced pressure, at 40°C. Calculate the weight of the lipid:

    Weight of lipid = (weight of container + extracted lipid) − (weight of container)
11. Calculate the lipid content by weight difference, as given below:

    Lipid content (%) = amount of lipid extracts (gram)/weight of original sample (gram) × 100

n-PROPANOL/WATER EXTRACTION

BASIC PROTOCOL 3

Lipids in starchy foods may occur in the free as well as bound forms. The latter being either in the form of amylose inclusion complexes or linked via ionic or hydrogen bonding to the hydroxyl groups of the starch components. Free lipids are easily extractable at ambient temperatures, while use of nonalcoholic solvents for a prolonged period or disruption of the granular structure by acid hydrolysis (see Basic Protocol 4) may be required for the efficient extraction of bound lipids. While acid hydrolysis allows the release and quantitation of lipids, the procedure leads to destruction of the starch components; therefore, the alcohol extraction system involving propanol and water would be most desirable in these cases. This system removes both nonpolar and polar lipids from samples.

Materials

Cereal sample (e.g., wheat, barley) or potato starch
3:1 (v/v) *n*-propanol/water: mix well prior to use
Deionized H_2O

Coffee grinder (optional)
Soxhlet apparatus (see Basic Protocol 1)
Rotary evaporator

1. Grind a sample of cereal, such as wheat or barley, in a coffee grinder, or use potato starch.
2. Accurately weigh 5 to 10 g of sample into a cellulose extraction thimble. Cover the top with glass wool to prevent floating.
3. Weigh the thimble and its contents. Do the same with the flat-bottom flask containing a few boiling chips.
4. Add 150 to 200 ml 3:1 (v/v) *n*-propanol/water to the Soxhlet apparatus.
5. Heat the *n*-propanol/water to the boiling point (~85°C). Allow the continuous extraction to proceed for 8 to 12 h.
6. Remove the solvent using a rotary evaporator under vacuum. Calculate the weight of the lipid:

   Weight of lipid = (weight of container + extracted lipid) − (weight of container)

7. Calculate the lipid content by weight difference as given below:

   Lipid content (%) = mass of lipid extracted (g)/weight of the original sample (g) × 100

EXTRACTION OF LIPIDS REQUIRING ACID DIGESTION

BASIC PROTOCOL 4

To quantitate total lipids, defined as the sum of the free and bound lipids, both polar and nonpolar, acid hydrolysis may be necessary to release the bound lipids by dissociating lipid-starch and lipid-protein intermolecular forces. The resultant lipids may then be removed and measured; however, the nonlipid components so obtained are not usable for further analysis. Removal of some of the polar lipids may hinder the use of the extracted material for further analysis.

Materials

Starch or fish meal
6 N HCl
Sodium sulfate, anhydrous
n-hexane or 1:1 (v/v) chloroform/methanol

Coffee grinder
250-ml round-bottom flask
Whatman no. 1 filter paper and funnel
Rotary evaporator

1. Grind a sample of starch or fish meal into a powder in a coffee grinder.
2. Weigh 5 g of the ground sample into a 250-ml round-bottom flask.
3. Hydrolyze the sample of starch with 50 ml 6 N HCl at 70° to 80°C for 1 hr or the sample of fish meal at 110°C for 4 to 24 hr, until complete dissolution.

   The temperature can be controlled using a water or oil bath.

4. Extract the total lipids 3 times with 50 ml each of *n*-hexane (starch) or 1:1 (v/v) chloroform/methanol (fish), retaining the organic layers each time.
5. Combine the organic layers and pass through a 2.5-cm thick layer of anhydrous sodium sulfate using Whatman no. 1 filter paper in a funnel, into a preweighed container suitable for a rotary evaporator.
6. Remove the organic solvent at 40°C under reduced pressure using a rotary evaporator. Calculate the weight of the lipid:

   Weight of lipid = (weight of container + extracted lipid) − (weight of container)

7. Calculate the content of lipids by weight difference, as given below:

   Lipid content (%) = mass of lipid extracted (g)/weight of original sample (g) × 100

COMMENTARY

Background Information

Lipids are one of the major components of food and are present even in so-called "fat-free" foods at low levels often in the bound form. Lipids are defined as materials that are sparingly soluble or insoluble in water, but soluble in organic solvents such as hexane, diethyl ether, benzene, chloroform, methanol, or their selected mixtures (Nawar, 1996; deMan, 1999). There are several classes of lipids, all having similar and specific characteristics due to the presence of a major hydrocarbon portion in their molecules. Over 80% to 85% of lipids are triacylglycerols, which occur in many types according to the identity and positions of the three fatty acids involved. Those with a single kind of fatty acid in all three are called simple triacylglycerols. As an example, trimyristin, isolated from nutmeg, is composed of myristic acid (C14:0) and glycerol. Triacylglycerols belong to the nonpolar class of lipids, which also includes diacylglycerols, monoacylglycerols, free fatty acids, and fat soluble vitamins, as well as steroids, terpenes, hydrocarbons, carotenoids, and other matter which cannot be saponified (Christie, 1982). Nonpolar lipids, also referred to as simple lipids, afford one or two types of products upon hydrolysis. On the other hand, polar lipids generally consist of phospholipids and glycolipids, and their hydrolysis may afford three or more products, hence they are also known as complex lipids. Phospholipids are major constituents of cell wall materials. An example of a plant phospholipid is lecithin, which is found in many vegetable oil sources. Glycolipids consisting of lipids in conjugation with carbohydrates are also found abundantly in biological systems and foods.

To analyze lipids, it is necessary to first isolate them quantitatively from nonlipid components. Extraction of lipids from source materials, such as food, animal and plant tissues, or microorganism, should be carried out in a manner that avoids changes in the lipids or leads to the formation of artifacts. Thus, it might be necessary to deactivate enzymes that might hydrolyse lipids via heat treatment. Precaution must also be exercised to minimize oxidation of lipids, especially those containing polyunsaturated fatty acids. Use of an antioxidant (e.g., TBHQ)/antioxidant system (e.g., TBHQ/EDTA) might prove beneficial, particularly when dealing with the extraction of lipids with highly unsaturated fatty acids, such as

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Lipids in foods exist in both the free and bound forms. While free lipids are easily extracted, bound lipids may require pretreatment with concentrated acids prior to their extraction (see Basic Protocol 4).

During the extraction process three types of interactions are usually disrupted, these are: van der Waals forces in lipid-lipid, lipid-protein, and liquid-carbohydrate complexes; electrostatic and hydrogen bonding interactions between lipids and proteins; and covalent bonding between lipids, carbohydrates, and proteins (Robyt and White, 1987). The solvent of choice depends on the type of lipid and the interactions to be disrupted. Thus, neutral lipids may be extracted with nonpolar solvents, while phospholipids and glycolipids are extracted with more polar solvent mixtures (Shahidi and Wanasundara, 1998).

The extraction and measurement of lipids may require several steps, these include: (1) Pretreatment, including drying, size reduction, and possibly acid hydrolysis to release lipids. (2) Homogenization of the tissue in the presence of a solvent/solvent system. (3) Separation of liquids from solids. (4) Removal of nonlipid contaminants. (5) Removal of solvent and drying. (6) Calculating the content of lipids by weight difference.

Procedures for isolation and measurement of lipids in foods include exhaustive Soxhlet extraction with hexane or petroleum ether (AOAC, 1995; see Basic Protocol 1), chloroform/methanol (Hanson and Olley, 1963; Ambrose, 1969), chloroform/methanol/water (Folch et al., 1957; Bligh and Dyer, 1959; see Basic Protocol 2 and Alternate Protocol 2), acid digestion followed by extraction (see Basic Protocol 4), or, for starchy material, extraction with *n*-propanol-water (e.g., Vasanthan and Hoover, 1992; see Basic Protocol 3). Each method has its own advantages and disadvantages and successful measurement of lipid content is often dictated by the type of sample and extraction medium employed. Commercial extraction and preparation of edible oils are explained in the literature (Williams, 1997).

In the Soxhlet extraction, usually dry or nearly dried material is subjected to semicontinuous extraction with hexane or petroleum ether. The drawbacks of this method are the length of time required for extraction and the fact that polar and bound lipids are not removed, offset by the fact that no channeling occurs. The Goldfisch method, on the other hand, is a continuous process and somewhat faster than that of the Soxhlet procedure. In the *n*-propanol-water extraction procedure, using Soxhlet, bound lipids in amylose and starchy food may be nearly completely released and polar lipids also quantitated. Nonetheless, total extraction and measurement of lipids may require acid digestion with strong sulfuric acid or usually 6 to 8 M HCl to release the bound lipids prior to their extraction into a desirable solvent. Meanwhile, the method of Folch et al. (1957) and that of Bligh and Dyer (1959) use chloroform/methanol/water to isolate total lipids. Use of chloroform is not ideal, but the method is of general use in wet foods, especially meat and fish. Recently, a rapid and simple method involving chloroform/methanol/water for determination of lipid content of seafoods has been reported by Lee et al. (1996). In addition, extraction of lipids from small amounts of material for determination and subsequent use for fatty acid analysis or directly for fatty acid profiling has been reported (Divakaran and Ostrowski, 1989; Garces and Mancha, 1993; Lugue de Castro and Garcia-Ayuso, 1998; Isik et al., 1999). Other methods have compared extraction with chloroform-based solvents with hexane/isopropanol (Gunnlaugsdottir and Ackman, 1993). Use of acetone/water has also been examined (Dambergs, 1956).

In addition to the above, other solvent systems and methodologies, both direct and indirect, have been explored for extraction and/or quantification of total lipids from different foods. As an example, Röse-Gottlieb extraction methodology (AOAC, 1995; James, 1995) is employed for extraction of lipids from milk and Schmid Bondzynski-Ratzlaff (Werner-Schmid) extraction of lipids from cheese is commonplace (AOAC, 1995; James, 1995). In the Röse-Gottlieb method, the sample is first treated with a solution of 25% (w/v) ammonia and then extracted repeatedly in the ethanol, diethyl ether, and hexanes (petroleum ether). The combined extracts are then dried and reextracted into petroleum ether followed by solvent removal and measurement. In the Mojonnier (AOAC, 1995; James, 1995) procedure, the combined extracts are then weighed without purification. The Schmidt-Bondzynski-Ratzlaff procedure uses successive extractions with diethyl ether after digestion with acid or base. Recently, use of analytical supercritical fluid extraction (SFE) for determination of fat content has been reported (Feller and King, 1996).

The indirect procedures are used primarily for measurement of lipids without isolation. The application of wide-line nuclear magnetic

resonance (NMR) for determination of oil content in oilseeds and use of infrared spectroscopy for determination of lipid in milk products has been reported (e.g., Shahidi and Wanasundara, 1998). Meanwhile, use of refractometry, density measurements and other techniques for lipid determination have been reported (See Pomeranz and Meloan, 1994).

Critical Parameters and Troubleshooting

In Soxhlet extractions (see Basic Protocols 1 and 3), the amount of solvent must be sufficient to cover the thimble and have at least 50 ml solvent in the flask and may require an additional amount of solvent. After completion of extraction, it is essential to let the system cool and let all the solvent transfer to the flask. In addition, accurate weighing of flask is essential. Use of gloves to avoid fingerprints on flasks which may affect the weights is critical.

There may be problems associated with phase separation due to the formation of emulsion (see Basic Protocol 2). Addition of a salt solution (KCl or NaCl) and further standing in a cold room is recommended.

Total removal of the solvent from extracted lipids is necessary (see Basic Protocols 1 to 4). Often keeping of the extract in a forced-air oven for 1 to 2 h is helpful.

If extracted lipids are to be used for further analysis, it is recommended that a certain volume of the extract be removed and used for this purpose prior to employing any harsh conditions for the removal of solvents. In this case, accurate measurement of values is essential.

Anticipated Results

Results for lipid content depends very much on the source material and the protocol used. Thus, oilseeds such as mustard, canola, and flax may contain 38% to 42% lipid, while starch may contain ≤1% total lipid. The content of lipids in meat and seafoods may vary from 1% to 30%. Nutmeg contains 25% to 30% lipids.

Time Considerations

For extraction of lipids, using all four protocols, approximately 35 to 40 hr is required. The time required for the Soxhlet method (see Basic Protocol 1) is up to 12 hr, the chloroform/methanol/water extraction (Basic Protocol 2), up to 12 hr, *n*-propanol/water extraction (see Basic Protocol 3), 5 to 6 hr, and extraction of lipids requiring acid digestion (see Basic Protocol 4), 5 to 8 hr.

Each of the alternate procedures requires the same time or less than that of the original.

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Analysis of Fatty Acids in Food Lipids

Fatty acid compositional analysis of lipids is usually carried out by gas-liquid chromatography (GC). Lipids obtained from food samples have complex structures and often fall into the following classes: triacylglycerols, phospholipids, sphingolipids, and sterols. Sterols exist in both animal- and plant-originated foods in either a free form (e.g., free cholesterol) or esterified form (e.g., cholesterol esters). Generally, most of the fatty acids found in food lipids are covalently bound to either an alcohol (glycerol) via ester bonds (triacylglycerols, phospholipids, and sterol esters), or to a long-chain base (sphingosines) via amide bonds (sphingolipids). To analyze the fatty acid composition of food lipids, the complex lipids must be pretreated so that the individual fatty acids are available for chromatographic analysis. For this purpose, fatty acids in the complex lipids are converted to their corresponding methyl esters by various derivatization methods to make them volatile for GC analysis. Since the focus of this unit is fatty acid analysis, and fatty acids from sterol esters contribute only a small portion of the total fatty acid pool, issues typical to the analysis of sterol-bound fatty acids will not be discussed; however, it should be noted that the protocols described in this unit do not work well for fatty-acid esterified sterols.

There are basically two mechanisms to convert the fatty acids in a complex lipid to fatty acid methyl esters (FAMEs): methylation following hydrolysis of the fatty acids from the complex lipids, or direct transesterification. The first mechanism involves saponification (alkaline hydrolysis) in which the ester bond is cleaved between the fatty acid and the glycerol moiety (e.g., triacylglycerols and phospholipids) under heat and in the presence of an alkali (usually sodium hydroxide), followed by methylation performed in the presence of an acidic catalyst in methanol. Direct transesterification is usually a one-step reaction involving alkaline or acidic catalysts.

This unit describes two basic protocols and one alternate protocol for FAME derivatization, and one support protocol for lipid extraction. The derivatization procedures described in this unit are suitable for a small amount of sample, ranging from 2 to 50 mg, which is particularly compatible with GC analysis using today's popular capillary or megabore fused-silica columns. In the first protocol, FAMEs are prepared from lipid samples by sodium hydroxide saponification followed by boron trifluoride (BF_3)–catalyzed esterification (see Basic Protocol 1). This procedure is adopted from the official methods (AOCS, 1989; Firestone, 1995) with modification. Basic Protocol 1 is suitable for most lipid analyses. Since BF_3 and other acidic catalysts will change the conformation of conjugated dienoic fatty acids (e.g., conjugated octadecadienoic acids; conjugated linoleic acids; CLA), this method is not recommended for lipid samples having these special structures, such as dairy products and ruminant meat products.

FAMEs are prepared by sodium methoxide (alkaline)-catalyzed transesterification in the second protocol, (see Basic Protocol 2). This is a popular method used to analyze samples containing CLA and works only on fatty acids having ester bonds; free fatty acids and fatty acids in sphingolipids will not be methylated. The sodium methoxide protocol gives comparable results to Basic Protocol 1 if the sample contains a low level of or no free fatty acids. In Alternate Protocol 1, another alkaline-catalyzed transesterification method utilizing tetramethylguanidine (TMG) is described. This method should have the same application considerations as Basic Protocol 2. The investigator of the original publication for this method claimed that TMG works on both esterified and free fatty acids; however; in the authors' experience, and in published studies, TMG does not satisfactorily convert free fatty acids to their methyl esters. Generally, Basic Protocol 1, though it is a two-step

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reaction, takes less time to complete and requires less glassware compared to Basic Protocol 2. Whenever possible, Basic Protocol 1 is recommended for routine sample preparation of food lipid samples.

A support protocol on lipid extraction is enclosed in this unit for convenience. Lipids in food samples, except oils and fats, are bound to other food constituents, such as proteins and carbohydrates. The attracting forces between lipids and other macromolecules are van der Waals' attractions, hydrophobic and electrostatic forces, hydrogen bonding, and covalent bonding. In order to extract lipids from food samples, it is necessary to find solvents that can dissolve the lipids readily and eliminate the attracting forces between lipids and other compounds within the food matrix. Experience indicates that a 2:1 (v/v) chloroform/methanol mixture is an effective solvent combination for extracting lipids from a vast variety of samples. The chloroform/methanol method was first introduced by Folch et al. (1957) for lipid-rich biological samples.

CAUTION: All of the procedures introduced in this unit involve the use of volatile and toxic organic solvents, such as chloroform and methanol. To prevent the contamination of the laboratory atmosphere, experiments using these procedures should be performed in a fully functional fume hood.

IMPORTANT NOTE: To avoid contamination of samples by plasticizers, use only glass pipets, tubes, and beakers. Do not use plastic vessels or Parafilm. Use only Teflon-lined caps for all glass tubes and sample vials whenever organic solvents are involved. Teflon is practically chemically inert and is not affected by the chemicals used in these protocols.

NOTE: GC standards (Nu-Chek-Prep and Matreya) in the form of free fatty acids or triacylglycerols, need to be methylated prior to GC analysis. Standards that are already in FAME form do not need to be derivatized again; they are simply diluted to an appropriate concentration and subjected to GC analysis directly.

BASIC PROTOCOL 1

PREPARATION OF FATTY ACID METHYL ESTERS FROM LIPID SAMPLES CATALYZED WITH BORON TRIFLUORIDE IN METHANOL

In this method, lipid samples are first saponified with an excess of NaOH in methanol. Liberated fatty acids are then methylated in the presence of BF_3 in methanol. The resulting fatty acid methyl esters (FAMEs) are extracted with an organic solvent (isooctane or hexane), and then sealed in GC sample vials for analysis. Because of the acidic condition and high temperature (100°C) used in the process, isomerization will occur to those fatty acids containing conjugated dienes, such as in dairy and ruminant meat products, that contain conjugated linoleic acids (CLA). If CLA isomers are of interest in the analysis, Basic Protocol 2 or the Alternate Protocol should be used instead. Based on experience, this method underestimates the amount of the naturally occurring *cis-9, trans*-11 CLA isomer by ~10%.

The formulas for the chemical reactions involved in this protocol are outlined in Equation D1.2.1:

$$\text{Saponification: RCOO-R}' + \text{NaOH} \underset{100^\circ\text{C}}{\rightleftharpoons} \text{RCOO-Na} + \text{R}'\text{-OH}$$

$$\text{Esterification: RCOO-Na} + \text{CH}_3\text{OH} \underset{100^\circ\text{C}}{\overset{\text{BF}_3}{\rightleftharpoons}} \text{RCOO-CH}_3 + \text{NaOH}$$

Equation D1.2.1

Materials

Extracted lipid sample in organic solvent (see Support Protocol)
Nitrogen gas: high purity (<5 mg O_2/kg)
0.5 N NaOH: 2 g NaOH diluted to 100 ml in methanol; store up to 6 months at 2° to 8°C
10% to 14% boron trifluoride (BF_3) in methanol (Sigma or Supelco; also see recipe)
Isooctane or hexane
Anhydrous sodium sulfate: store at 100°C

N-EVAP model 112 nitrogen evaporator (Organomation) with moisture trap in line from N_2 source (Fig. D1.2.1)
15-ml test tubes with Teflon-lined screw caps
100°C heating block
Test tube shaker
Tabletop centrifuge
GC sample vials with Teflon caps

1. Using a nitrogen evaporator, dry the sample down completely under a stream of nitrogen at 40°C in a 15-ml glass tube with a Teflon-lined screw-cap.

   IMPORTANT NOTE: *Use high-purity nitrogen only, otherwise impurities of O_2 will oxidize the lipids (see Critical Parameters). A multiport nitrogen evaporator equipped with a distribution manifold and water bath is highly recommended for this application (Fig. D1.2.1).*

Figure D1.2.1 An example of a multiport (24-position) nitrogen evaporator (N-EVAP, Organomation Associates, reprinted with permission).

Lipid samples are generally dissolved in 2:1 (v/v) chloroform/methanol if the lipid is extracted using a protocol similar to the Support Protocol, as both polar and neutral lipids are soluble in this mixture.

2. Add 0.4 ml 0.5 N NaOH in methanol to the tubes and replace the Teflon-lined screw cap tightly. Place the tubes in a 100°C heating block for 5 min to saponify the lipid.
3. Remove tubes from the heating block and cool down with tap water.
4. Add 0.4 ml 10% to 14% BF_3 in methanol and cap the tubes. Place in a 100°C heating block for 5 min to methylate fatty acids.
5. Remove tubes from the 100°C heating block and cool down with tap water. Add the appropriate amount of solvent (i.e., isooctane or hexane) to extract the FAME.

   Adjust solvent volume depending on the amount of FAME present in the tube and the sample amount required for GC analysis. Generally, add 1 ml for samples with large amounts (20 mg) of FAME and 0.5 ml for small amounts (<5 mg).
6. Add 8.5 ml of distilled water. Cap the tubes and shake on a test tube shaker for 10 min at room temperature.

   Use a saturated NaCl solution instead of water if working with samples containing a significant amount of short-chain fatty acids, such as milk fat and coconut oil (Bannon et al., 1982).
7. Centrifuge the tubes for 5 min at 1000 × *g*, room temperature, in a tabletop centrifuge to separate the organic and aqueous phases.
8. Recover the upper organic layer containing the FAME. Place the organic layer in a 1.5 ml GC sample vial (Teflon cap) containing a ~1 mm-deep layer of anhydrous sodium sulfate on the bottom. Properly dispose of the lower layer that contains methanol.

   The anhydrous sodium sulfate removes water from the sample.
9. Analyze the sample by GC (see Critical Parameters).

   Flush the sample with N_2 gas and store at −20°C if not used immediately.

BASIC PROTOCOL 2

PREPARATION OF FATTY ACID METHYL ESTERS FROM LIPID SAMPLES CATALYZED WITH SODIUM METHOXIDE IN METHANOL

In this method, lipid samples are first dissolved in dry toluene, then treated with sodium methoxide, an alkaline catalyst. Fatty acids in complex lipids are then transesterified to form their corresponding methyl esters. This is a relatively mild reaction and does not produce isomerization of conjugated dienes. The limitation of this method is that, unlike the BF_3 method (see Basic Protocol 1), free fatty acids are not methylated and the procedure requires more time and glassware.

The formula for the chemical reaction involved in this protocol is outlined in Equation D1.2.2:

$$\text{Transesterification: } RCOO\text{-}R' + CH_3OH \underset{50^oC}{\overset{NaOMe}{\rightleftharpoons}} RCOO\text{-}CH_3 + R'\text{-}OH$$

Equation D1.2.2

Materials

Extracted lipid sample in organic solvent (see Support Protocol)
Nitrogen gas: high purity (<5 mg O_2/kg)
Toluene, dry
0.5 N sodium methoxide in methanol (Sigma; also see recipe)
Glacial acetic acid
Saturated (360 g/liter) NaCl in distilled H_2O
Hexane
Anhydrous sodium sulfate: store at 100°C

15-ml test tube with a Teflon-lined screw cap
N-EVAP model 112 nitrogen evaporator (Organomation) with moisture trap in line from N_2 source
50°C heating block
Test tube shaker
Tabletop centrifuge
GC sample vials with Teflon caps

1. Using a nitrogen evaporator, dry the sample down completely under a stream of nitrogen at 40°C in a 15-ml glass tube with a Teflon-lined screw-cap.

   IMPORTANT NOTE: *Use high-purity nitrogen only, otherwise impurities of O_2 will oxidize the lipids (see Critical Parameters). A multiport nitrogen evaporator equipped with a distribution manifold and water bath is highly recommended for this application (Fig. D1.2.1).*

   Lipid samples are generally dissolved in 2:1 (v/v) chloroform/methanol if the lipid is extracted using a protocol similar to the Support Protocol, as both polar and neutral lipids are soluble in this mixture.

2. For each sample, dissolve the dried lipid in 1 ml of dry toluene in a 15-ml test tube with a Teflon-lined screw-cap. Add 2 ml of 0.5 N sodium methoxide in methanol and cap tightly.

   Since nonpolar lipids (e.g., triacylglycerols) are generally not readily soluble in methanol, a solvent that can dissolve both neutral and polar lipids is necessary to facilitate the reaction in methanol. Toluene is chosen for this purpose because of its good solvent properties and relatively low toxicity.

3. Heat the tubes for 10 min in a 50°C heating block.

4. Add 0.1 ml of glacial acetic acid followed by 5 ml of saturated NaCl solution to the tubes.

5. Add 3 ml of hexane. Agitate the tubes for 10 min on a test tube shaker. Centrifuge the tubes for 10 min at $1000 \times g$, room temperature, in a tabletop centrifuge to separate the organic and aqueous layers.

6. Transfer the upper organic (i.e., hexane) layer into a clean, labeled glass tube with a ~1 mm-deep layer of anhydrous sodium sulfate on the bottom. Extract the lower aqueous layer from the original extraction again as described in step 5 to remove residual FAME.

   The anhydrous sodium sulfate removes water from the sample.

7. Transfer the upper hexane layer to the tube containing the first hexane layer.

8. Remove hexane from the methylated fatty acids by placing the tubes in the N-EVAP model 112 nitrogen evaporator under mild water bath conditions (40° to 45°C), evaporating the organic solvent under a gentle stream of nitrogen gas (Fig. D1.2.1).

   Dry lipid may still appear wet even when hexane is removed. Overdrying can result in loss and degradation of the sample.

9. Reconstitute the FAME in a volume of hexane appropriate for the conditions of GC analysis (see Critical Parameters) and FAME concentration. Transfer to GC sample vials with Teflon caps and perform GC analysis.

ALTERNATE PROTOCOL

PREPARATION OF FATTY ACID METHYL ESTERS FROM LIPID SAMPLES CATALYZED BY TETRAMETHYLGUANIDINE (TMG)

This is another alkaline-catalyzed transesterification technique. Similar to the sodium methoxide method (see Basic Protocol 2), only one step is required for the methylation reaction, followed by FAME extraction and isolation. As opposed to the sodium methoxide method, the investigator of the original publication for this method claimed that the tetramethylguanidine (TMG) works on both esterified and free fatty acids; however, in the authors' experience, and in published studies (see Background Information), TMG does not satisfactorily convert free fatty acids to corresponding methyl esters.

The formula for the chemical reaction involved in this protocol is outlined in Equation D1.2.3:

$$\text{Transesterification: } RCOO\text{-}R' + CH_3OH \underset{100^\circ C}{\overset{TMG}{\rightleftharpoons}} RCOO\text{-}CH_3 + R'\text{-}OH$$

Equation D1.2.3

Additional Materials *(also see Basic Protocol 2)*

1:4 (v/v) tetramethylguanidine (TMG)/methanol: store up to 3 months at 2° to 8°C
100°C heating block

1. Using a nitrogen evaporator, dry the sample down completely under a stream of nitrogen at 40°C in a 15-ml glass tube with a Teflon-lined screw-cap.

   See Basic Protocol 2 for additional comments on this step.

2. Add 1 ml of 1:4 (v/v) TMG in methanol using a disposable glass pipet and cap the test tube.

3. Heat the tubes for 10 min in a 100°C heating block. Remove the tubes and cool in a shallow water bath at room temperature.

4. Add 8 ml of saturated NaCl and 3 ml of hexane to each tube, shake for 10 min, and centrifuge for 10 min at 1000 × *g*, room temperature, in a tabletop centrifuge to separate the organic and aqueous layers.

5. Transfer the upper organic (hexane) layer into a clean, labeled glass tube with a ~1 mm-deep layer of anhydrous sodium sulfate on bottom.

6. Add a second 3-ml aliquot of hexane to the remaining lower aqueous layer. Shake for 10 min, then centrifuge for another 10 min at 1000 × *g*, room temperature, in a tabletop centrifuge.

7. Transfer the upper hexane layer to the tube containing the first hexane layer and the ~1 mm anhydrous sodium sulfate layer.
8. Remove hexane from the FAME by evaporating under a gentle stream of nitrogen gas (Fig. D1.2.1).
9. Dissolve the resulting FAME in 1 ml of isooctane (or a volume appropriate for the sample concentration) and transfer to a GC sample vial for analysis (see Critical Parameters).

LIPID EXTRACTION UTILIZING CHLOROFORM-METHANOL

SUPPORT PROTOCOL

This method is a modified Folch procedure (Folch et al., 1957), utilizing 2:1 (v/v) chloroform/methanol as the organic solvent. Lipid samples are homogenized vigorously in methanol followed by a mixture of chloroform/methanol, and the extracted lipids are filtered into a collection tube. A 0.88% KCl aqueous solution is added to the lipid extracts, followed by vigorous shaking to wash the extracts. After the aqueous wash, the extracts are allowed to become biphasic in a refrigerator overnight or immediately centrifuged to accelerate the separation of the organic and aqueous phases. The lower organic phase containing mostly chloroform and the extracted lipids is then recovered. This method is especially suitable for processing a large number of samples in a relatively short time (see Time Considerations).

Materials

Sample, fresh or frozen
Methanol (HPLC grade)
Chloroform (HPLC grade)
2:1 (v/v) chloroform/methanol (HPLC grade)
0.88% (8.8 g/liter) KCl in distilled H_2O
Nitrogen gas: high purity (<5 mg O_2/kg)

Centrifuge tube with spout or 50-ml glass tube
Polytron PT-3100 homogenizer equipped with a PT-DA 3012/2EC aggregate (probe) or equivalent
Whatman no. 40 filter paper
15- and 50-ml test tubes with Teflon-lined screw cap
Test tube shaker
Tabletop centrifuge
Vacuum aspirator
N-EVAP model 112 nitrogen evaporator (Organomation) with moisture trap in line from N_2 source
Glass pipets
Glass beakers

1. Weigh out 0.5 to 1.0 g fresh or frozen sample into a centrifuge tube with a spout or a 50-ml glass tube. Immediately add 7 ml of methanol.

   Plant oils and animal fat samples (vegetable oils, tallow, lard, fish oils) do not need to be extracted before methylation. A small amount of these samples (10 to 20 mg) can be subjected to the methylation procedures directly.

2. Homogenize the sample for 30 sec with a Polytron PT-3100 homogenizer.

   Clean the homogenizer generator probe between samples. Run homogenizer for several seconds in 2:1 (v/v) chloroform/methanol, followed with distilled water to clean. Wipe probe clean and dry. Change rinse solutions after every four samples or as needed.

3. Add 14 ml of chloroform and repeat homogenization as described in step 2 for 30 sec.

   The time needed for homogenization varies based on the nature of the sample. For some hard-to-break samples, pretreatment with liquid nitrogen and pulverization will help.

4. Filter the homogenate through Whatman no. 40 filter paper into a 50-ml glass tube with Teflon-lined screw-cap.

5. Rinse original tube with an additional 12 ml of 2:1 (v/v) chloroform/methanol and homogenize for another 5 sec to rinse the probe. Pour the solvent back into the original funnel lined with filter paper as described in step 4.

6. When filtration is complete, add 8 ml of 0.88% KCl to the glass tube and cap securely.

   It is important to keep the solvent ratio as close as possible to 8:4:3 (v/v/v) chloroform/methanol/water, to prevent selective loss of lipid classes during this step (Christie, 1989a). Therefore, it is necessary to estimate the water content of the lipid sample before extraction and take this into consideration when determining the amount of 0.88% KCl needed. In addition, during filtration, a portion of the organic solution is lost due to evaporation directly from the funnel, increasing with filtration time. One should try to estimate the loss and adjust the volume of the 0.88% KCl based on empirical observation for a specific type of sample.

7. Shake the tube on a test tube shaker for 10 min.

8. Centrifuge the tube for 5 min at $1000 \times g$, room temperature, in a tabletop centrifuge, or refrigerate overnight to allow the sample to become biphasic.

   Lipids will remain in the lower organic layer.

9. Remove the upper, aqueous layer by vacuum aspiration.

10. Using a nitrogen evaporator, evaporate the chloroform layer under a stream of nitrogen until ~3 ml remains.

    IMPORTANT NOTE: *Use high-purity nitrogen only, otherwise impurities of O_2 will oxidize the lipids. A multiport nitrogen evaporator equipped with a distribution manifold and water bath is highly recommended for this application (Fig. D1.2.1).*

11. Transfer the concentrated sample to a 15-ml glass tube with Teflon-lined screw-top. Rinse the 50-ml tube with 2 ml 2:1 (v/v) chloroform/methanol and transfer rinses to the 15-ml tube.

12. If the extracted lipid is to be stored, flush the tube with nitrogen gas and tightly close the cap, and leave at −20°C for future applications. Otherwise, proceed to the methylation technique of choice (see Basic Protocol 1 and 2; also see Alternate Protocol).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Boron trifluoride (BF_3) in methanol, 10% to 14% (w/v)

Prepare by bubbling BF_3 gas into methanol. Store up to 2 years at 4°C.

CAUTION: *The use of commercially prepared BF_3 reagent (Sigma or Supelco) is strongly recommended because of the high toxicity of gaseous BF_3.*

Detailed procedures are described in Firestone (1995).

Sodium methoxide in methanol, 0.5 N

Prepare by dissolving 0.5 mol Na metal in methanol. Store up to 2 years at 4°C (Firestone, 1995).

CAUTION: *The use of commercially available sodium methoxide (Sigma or Aldrich) is recommended as Na metal is highly corrosive and will explode upon contact with water.*

COMMENTARY

Background Information

Sample preparation is probably the most important step in any analytical procedure. Poor preparation of lipid samples will only yield inferior or questionable results. Some commonly performed sample-preparation procedures for gas-liquid chromatographic (GC) analysis of fatty acids in food samples are introduced in this unit. Since the introduction of gas chromatography in the 1950s, significant progress has been made in fatty acid analysis of lipids; however, fatty acid methyl esters (FAMEs) are still the most commonly used fatty acid derivative for routine analysis of food fatty acid composition.

Various methods are currently available for the preparation of FAMEs. Both acid and alkaline catalysts can be used for this purpose. Examples of the acidic catalysts include 10% to 14% (w/v) BF_3 in methanol (see Basic Protocol 1), 10% (w/v) BCl_3 in methanol, 5% (w/v) anhydrous HCl in methanol, and 1% to 2% (v/v) concentrated sulfuric acid in methanol. Examples of basic catalysts include 0.5 N sodium methoxide (see Basic Protocol 2) and 1:4 (v/v) tetramethylguanidine (TMG) in methanol (see Alternate Protocol).

There are advantages and drawbacks for each of the aforementioned reagents. No single method can fulfill the methylation requirement for all kinds of samples. The BF_3 method (see Basic Protocol 1), since its introduction by Metcalfe et al. (1961, 1966), has been widely used and is adopted as the official method for both the American Oil Chemists' Society (methods Ce 2-66 and Ce 1b-89; AOCS, 1989) and AOAC International (969.33; Firestone, 1995). The advantage of the BF_3 method is that it methylates free fatty acids quickly, and if combined with a saponification step, as in the official methods (AOCS, 1989; Firestone, 1995) and Basic Protocol 1, it methylates esterified fatty acids quickly as well. It has been reported that when using a past-date or too concentrated reagent, production of artifacts and loss of polyunsaturated fatty acids may result (Christie, 1989a). Since this reagent is readily available from commercial sources in sealed glass ampules (i.e., O_2-depleted), the freshness and proper concentration can be practically guaranteed. The shelf life, when made fresh and stored properly, can be as long as two years (Firestone, 1995). The use of other acidic catalysts is not recommended because the reactions take a longer time to complete and the reagents must be prepared often, since they have a relatively short shelf life. For example, sulfuric acid in methanol is only stable for 1 to 2 days, while the concentration of the HCl in methanol reagent is not easily controlled, and potential hazards exist when using corrosive HCl gas for preparation.

On the other hand, BF_3 (see Basic Protocol 1), as well as other acidic catalysts, will change the double-bond configuration of fatty acids that contain conjugated dienes. As research on conjugated linoleic acid (CLA) and other conjugated fatty acids becomes more popular, it is essential not to provide misinformation about compositional analysis due to improper application of a methylation protocol (Li and Watkins, 1998). The basic catalysts perform better on lipids rich in fatty acids with unique conjugated diene structures. Isomerization and artifacts are not produced when sodium methoxide or TMG are used as transesterification agents

(see Basic Protocol 2 and Alternate Protocol). However, they do not methylate free fatty acids and N-linked (amide-bond) fatty acids such as those found in sphingolipids. Though the original author of the TMG method (Schuchardt and Lopes, 1988) claimed that it worked fine with free fatty acids, recent tests by other researchers showed different results. Those reports conclude that TMG is not effective in methylating free fatty acids and phospholipids (Shantha et al., 1993; Kramer et al., 1997). Therefore, these methods are not suitable for samples with high acid values. For CLA analysis in samples having high levels of free fatty acids, Kramer et al. (1997) reported a method using both acidic and basic catalysts in sequence. This method resulted in less CLA loss than using acid-catalyzed methylation alone, as well as a complete methylation of free fatty acids. Generally, there is no single method that works optimally in all situations. The investigator should know the nature of the sample and select the appropriate method accordingly.

One method that uses none of the above catalysts is worthy of mention, the diazomethane method. This method is very useful if there is only a very limited amount of free fatty acids as the starting material, since no extraction is needed before the resulting FAME can be injected into the GC for analysis. The authors of this unit have never performed this method. Interested readers can learn more from information presented elsewhere (Christie, 1989b). In addition, esters other than methyl may be required from time to time for specific purposes, and this is beyond the scope of the present discussion.

A protocol on lipid extraction modified from the pioneering work of Folch et al. (1957) is included in this unit for convenience (see Support Protocol). The authors' laboratory uses this protocol for virtually all kinds of samples, and have found it to be effective with reproducibly high recoveries. Another very popular lipid extraction technique is the Bligh and Dyer method, which uses a 1:1 (v/v) mixture of chloroform/methanol (Bligh and Dyer, 1959). For more information on lipid extraction, the discussion by Nelson (1991) on solvent selection and extraction efficiency is recommended.

Critical Parameters

Settings in a GC system, the primary tool for fatty acid analysis, will affect the results of analysis greatly. A GC system has the following elementary parts: carrier gas supply, injector, column, and detector.

Carrier gases

Carrier gas brings the injected FAME sample through the column into the detector. It also serves as the mobile phase in the chromatographic system. The nature and the velocity of the carrier gas can affect the column efficiency significantly, as measured by the height of an effective theoretical plate. Helium and hydrogen are both very good sources of carrier gas; however, hydrogen is superior to helium, as column efficiency varies much less over a broad range of gas velocities with hydrogen, and therefore careful control of the velocity of carrier gas is less critical when hydrogen is used. In practice, however, because of the explosive nature of hydrogen with oxygen in the atmosphere, when using it as the carrier gas, caution should be taken to prevent hydrogen buildup by either maintaining good ventilation, or by using a hydrogen trap to retain hydrogen gas from all sources (e.g., split vent and purge vent).

Columns

The column is the most critical part of a GC system and is chosen based on the nature of the analysis. There are two types of columns available for GC analysis: packed columns and capillary (e.g., wall coated open tubular; WCOT) columns. A packed column is not the primary choice for today's routine analysis of fatty acid composition due to its low resolution and requirement for large amounts of sample. Compared to a packed column, a capillary column needs far less sample and is able to achieve superior resolution.

Capillary columns are made from fused silica and are coated with a thin uniform liquid phase inside the column as the stationary phase. The liquid phase can also be bonded chemically to the silica surface to enhance the stability of the column. There are many kinds of coating materials, and they can be classified generally as nonpolar, polar, and high-polar. For FAME analysis, polar and high-polar columns are preferred because of the presence of unsaturated fatty acids and their positional isomers which have high polarity relative to their saturated counterparts. Physical characteristics of the column will also have an impact on the analysis. Even for columns with the same coating material, factors such as column length, internal diameter (i.d.), and film thickness of the coating material will affect the performance of the column. Therefore, when selecting a column, one should not only consider the type of column for a particular assay, but also the physical charac-

Table D1.2.1 Information on Recommended Gas Chromatography Columns for FAME Analysis

Sample type	Column recommended	Dimensions available[a]
General analysis of plant oils and meat products	DB23 (J&W Scientific)	i.d.: 0.18, 0.25, 0.32, and 0.53 mm Length: 10, 15, 20, 30, 40, and 60 m
	DB225 (J&W Scientific)	i.d.: 0.05, 0.10, 0.18, 0.20, 0.25, 0.32, 0.45, and 0.53 mm Length: 10, 12, 15, 20, 25, and 30 m Film thickness: 0.05 to 1.0 µm
	PAG (Supelco)	i.d.: 0.20, 0.32, and 0.53 mm Length: 15, 30, and 60 m Film thickness: 0.25 and 0.50 µm
Food samples containing fish oil	DB23 (J&W Scientific)	i.d.: 0.18 - 0.53 mm Length: 10 - 60 m Film thickness: 0.15-0.5 µm
	SPB-PUFA (Supelco)[b]	i.d.: 0.25, 0.32 mm Length: 30 m Film thickness: 0.20 µm
	Omegawax (Supelco)[b]	i.d.: 0.25, 0.32, 0.53 mm Length: 30 m Film thickness: 0.25 and 0.50 µm
Food samples containing geometric-positional (*cis*/*trans*) isomers of fatty acids	Supelcowax-10 (Supelco)	i.d.: 0.10, 0.20, 0.25, 0.32, 0.53 mm Length: 5, 10, 15, 30, 60, 100 m Film thickness: 0.10, 0.15, 0.20, 0.25, 0.50, 1.0, and 2.0 µm
	Omegawax (Supelco)	i.d.: 0.25, 0.32, 0.53 mm Length: 30 m Film thickness: 0.25 and 0.50 µm
	SP-2560 (Supelco)[c]	i.d.: 0.25 mm Length: 100 m Film thickness: 0.20 µm
	SP-2380 (Supelco)	i.d.: 0.25 mm Length: 100 m Film thickness: 0.20 µm
	DB-Wax (J&W Scientific)	i.d.: 0.05, 0.1, 0.18, 0.2, 0.25, 0.32, 0.45, 0.53 mm Length: 5, 10, 20, 15, 25, 30, 40, 60 m Film thickness: 0.05, 0.10, 0.18, 0.20, 0.25, 0.30, 0.40, 0.42, 0.50, 0.85, 1.00, 1.70, and 2.0 µm
	CP-Sil 88 (Chrompack)	i.d.: 0.25, 0.32 mm Length: 25, 50 m

[a]Columns may not be available with all possible combinations of the i.d.; film thickness is stationary phase thickness.

[b]Recommended by manufacturer for PUFA analysis.

[c]Recommended by manufacturer for assay of hydrogenated vegetable oil.

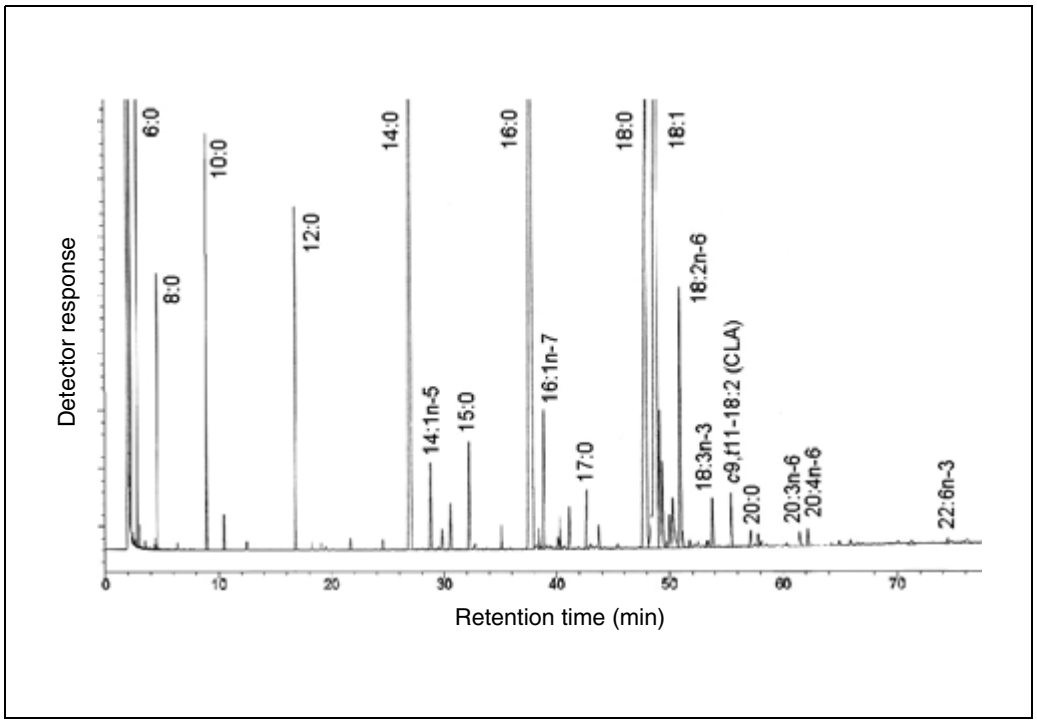

Figure D1.2.2 Sample GC chromatogram of the FAME from butter fat (Sweet Cream Butter, Wisconsin Grade AA, Roundy's, Milwaukee, Wisc.) prepared using the sodium methoxide method (see Basic Protocol 2). Equipment: DB-23 fused silica capillary column, 30 m × 0.32 mm i.d., 0.25 μm film thickness, FID detector. Temperature, injector: 225°C; detector: 250°C. Column (oven) temperature program: 100°C initial, hold 4 min, ramp to 198°C at 1.5°C/min, hold 10 min. Total run time was 80 min. Split injection.

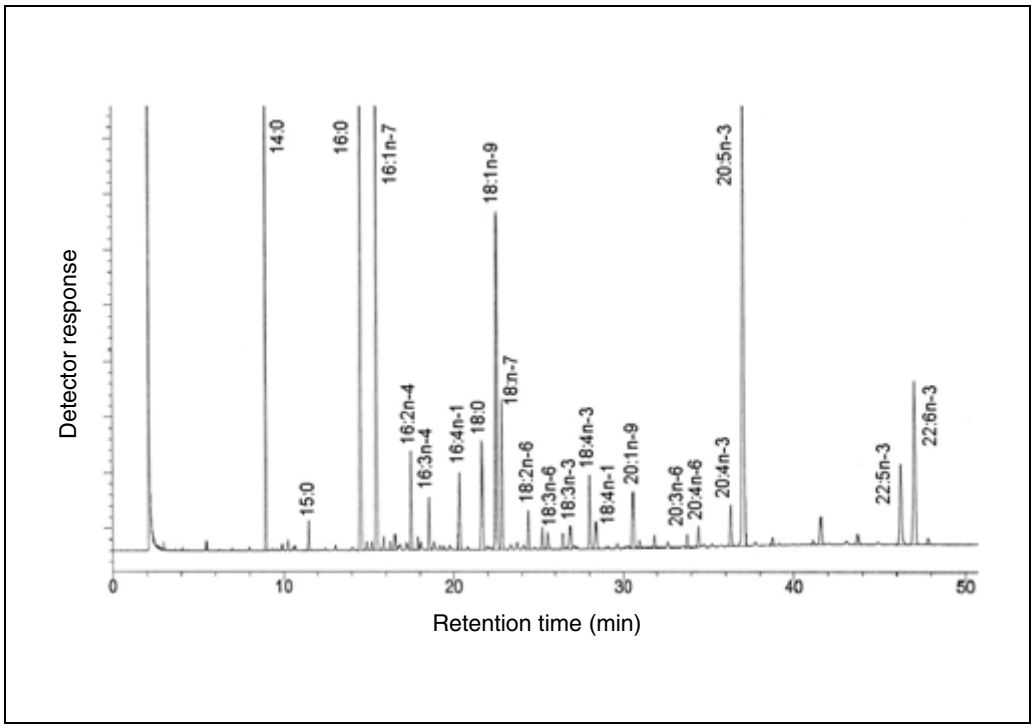

Figure D1.2.3 Sample GC chromatogram of the FAME of fish oil (menhaden oil) prepared using the boron trifluoride method (see Basic Protocol 1). Equipment: DB-23 fused silica capillary column, 30 m × 0.32 mm i.d., 0.25 μm film thickness, FID detector. Temperature, injector: 225°C; detector: 250°C. Column (oven) temperature program: 140°C initial, hold 2 min, ramp to 198°C at 1.5°C/min, hold 20 min. Total run time was 60 min. Split injection.

teristics of the column. Table D1.2.1 lists columns commonly used by investigators across disciplines to obtain the fatty acid compositions of a variety of lipid samples. Investigators are encouraged to speak to the column manufacturers for detailed information and suggestions on column selection and operating conditions for a particular application.

Injection techniques

There are three injection techniques for introducing a sample into a GC equipped with a capillary column: split injection, splitless injection, and on-column injection. Split injection is the most often used injection technique. When a certain amount of FAME sample (1 to 3 μl) is introduced into the GC injector that is normally set at a temperature much higher than the boiling point of the solvent, the solvent vaporizes instantly in the carrier gas and creates a large volume of gas that contains all of the injected FAME in it. The carrier gas that contains the FAME is then divided into two streams from the injector: one is directed onto the column, and the second is vented to the atmosphere, clearing the sample out of the injection chamber momentarily. This way, only a limited amount of sample is introduced into the column, to avoid column overloading, and injection time is short, to avoid peak broadening.

Splitless injection is used when the sample is dilute and cannot be introduced into the GC system with stream splitting. In practice, the column temperature is set 10° to 30°C below the boiling point of the solvent at the time of injection. When sample is introduced into the injector inlet, vaporized solvent together with the FAME condense at the beginning of the column along with the carrier gas flow. The condensed solvent plus the stationary phase of the column forms a diluted stationary phase that traps the FAME in it. After the initial sample introduction period, the column temperature is raised to normal operating conditions, and chromatographic separation starts from there.

Dilute samples can also be introduced into the GC system by on-column injection; however, this technique requires special syringes or other devices and therefore will not be discussed in detail. Split or splitless injection, on the other hand, can be performed in a GC system equipped with a split/splitless injector without the need for additional devices. Investigators can simply change the control of the split/splitless valve and the oven temperature for the column to choose between the two injection techniques.

Standards

Once introduced into the column, FAMEs with different carbon chain length and saturation levels move through the column at different rates and elute from the end of the column sequentially. Fatty acid standards (Nu Chek Prep or Matreya) are required to obtain the retention time for individual fatty acids in GC analysis, so that fatty acids in samples can be identified by comparing their retention times with those of the standards. In a GC run of multiple samples, standards should be analyzed prior to, during, and at the end of the sample analysis to compensate for shifts in retention times.

Flame ionization detectors

The end of the column is attached to a detector that serves to pick up signals from the eluates. A commonly used detector for FAME analysis is the flame ionization detector (FID). An FID operates by converting the carbon in organic compounds to carbon ions with a hydrogen flame. The signal (ion current captured by the detector) from the ionized carbons is then collected and amplified by the detector, and plotted into chromatograms by the control computer. Additional gas supplies—hydrogen, air, or most often nitrogen—are required for a FID to work normally, because of the low flow rate of the carrier gas through the column. Nitrogen serves as a makeup gas to accelerate the flow rate of column eluates, and helps to bring the FAME molecules to the detector. The response of the FID is determined by the weight percent content of ionizable (not oxidized) carbon atoms; therefore, response is not truly linear relative to the weight of the individual fatty acids in the sample. When calculating the fatty acid composition by weight percent, a correcting factor, called theoretical relative response factor (TRF; Ackman, 1991), should be used to multiply the corresponding peak areas obtained by the data acquisition equipment.

Contamination

Precautions should be taken to prevent oxidation during lipid analysis. Polyunsaturated fatty acids in lipid samples are easily attacked by active oxygen species (e.g., free radicals), exacerbated by the presence of strong light and metal ions. Therefore, it is a rule of thumb while working with lipids that samples should be handled in a way that minimizes contact with air, light, and metals. To accomplish this, handle samples in glass vessels, use Teflon-lined or coated materials, and maintain the samples

in an atmosphere of nitrogen. Whenever a sample is going to be stored for some time, flush the glass tube with nitrogen gas, close the Teflon-lined screw cap tightly, and store it at –20°C.

Another precaution is to avoid the use of plastic materials during storage, preparation of samples, and analysis, because plasticizers can contaminate the sample when organic solvents are present. As a safety concern, since there is no way to avoid exposure to organic solvents used in these procedures, all work should be performed in a fume hood to maintain a safe working environment.

Anticipated Results

The quality of FAME prepared by the methods described in this unit must be examined by GC analysis. Generally, impurities in the extracted lipid samples are not removed before methylation. If the GC results are not satisfactory due to sample contamination, additional steps may be necessary to clean the sample either before or after methylation. Commonly used techniques for purifying lipid samples are thin-layer chromatography (TLC), solid phase extraction (SPE), and column chromatography.

The configuration of the GC system also affects the result. Many factors, such as column type, gas flow control, and temperature programming, if not set up correctly, will affect the performance of the GC. In the authors' opinion, the first thing to do in a GC analysis is to choose the right column. One can then elucidate the optimal conditions for other factors (see Critical Parameters).

There are many GC columns available for FAME analysis (see Critical Parameters). Generally, a good column for FAME should be able to separate all the components and allow the FAME to elute primarily according to carbon chain length and secondarily by the number of double bonds. There should be no overlapping or minimal overlapping among FAME having different chain lengths. Table D1.2.1 lists GC columns commonly used for FAME analysis.

For most of the columns listed in Table D1.2.1, various dimensions are available for each of the column types. Generally, a column with an i.d. from 0.20 to 0.32 mm is regarded as a capillary column. A column with an i.d. of 0.45 or 0.53 mm is called a megabore column. Both capillary and megabore columns are commonly used in FAME analysis. There are advantages and disadvantages for both capillary and megabore columns. Capillary columns have better resolution but less sample capacity, and are subject to overloading. Megabore columns give satisfactory separation most of the time, and are more resistant to overloading; therefore samples prepared for a megabore column do not need to be as concentrated as those prepared for capillary columns. In practice, the authors recommend using a column that has been adopted by a majority of researchers for the application of interest. This way, one can easily compare his/her results with the published data.

Two sample chromatograms using the same column but different temperature programming are presented in Figures D1.2.2, and D1.2.3 illustrating analysis of commercial butter fat and fish oil. Fatty acids with carbon-chain length of six or more are clearly separated and integrated. Butyric acid (4:0) was resolved, but not integrated. Figure D1.2.3 shows an analysis of menhaden oil. The polyunsaturated fatty acids (PUFA) with various chain lengths and unsaturation levels are satisfactorily resolved in this chromatogram.

Time Considerations

Other than the time specified in the individual steps of each protocol, the total time spent on each methylation procedure depends largely on the sample size, the time required for evaporation of organic solvents, and the availability of space for sample tubes in the heating block and the manifold of the nitrogen-stream evaporator.

The BF_3 method takes ~40 min to complete. If a large number of samples must be processed, the time will be longer because of the time needed between the experimental steps, such as transferring tubes and pipetting solvents, and space limitations of the heating block and evaporator.

Both sodium methoxide and TMG methods require ~1 to 1.5 hr to complete; however, the actual time required depends on the sample size and the time used to evaporate the hexane from the FAME extracts.

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This book provides a practical guide to various aspects of lipid analysis, covering topics from sample preparation (extraction, fractionation, and derivatization) to GC analysis. Various derivatization methods are discussed and specific procedures are given for each of them. The book provides a comprehensive overview of GC technology including instrumentation (i.e., column, oven, carrier gas, injector, and detector) and data collection.

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Cholesterol

This unit describes the methods for quantitative determination of cholesterol in food and foodstuffs by chromatography and by an enzymatic determination using spectrophotometry. Two types of lipid classes including free cholesterol and cholesterol ester exist in food. Total cholesterol is measured after chemical hydrolysis of cholesterol esters to free cholesterol. In a similar way, fatty acids can be analyzed by gas chromatography (GC). Although successful results can be obtained by high-performance liquid chromatography (HPLC) without any derivatization of cholesterol, more sensitive and specific determination can be achieved by chemical conversion of cholesterol to the corresponding derivatized form. Since food and foodstuffs contain cholesterol and many sterol analogs, including phytosterols and oxidized cholesterol, appropriate chromatographic separation of cholesterol itself should be required for accurate quantification. The HPLC system required for cholesterol quantification is widely available in laboratories; however, it may be difficult to obtain a complete isolation of cholesterol by HPLC. GC is the most adequate method for this purpose and has been generally applied for quantitative measurement of cholesterol. Trimethylsilyl (TMS) ether derivatization is necessary before GC analysis. Colorimetric determination of cholesterol by chemical coloration of anisidine has been widely used and is one of the traditional methods. However, this measurement is not specific to cholesterol but also detects other corresponding analogs, and is therefore not covered in this unit. Enzymatic measurement is more specific to cholesterol compared to the colorimetric method, and expensive analytical instruments are not required. Lipid extraction prior to determination is not required in the enzymatic analysis. However, the results obtained by enzymatic measurement for certain foodstuffs are usually overestimated compared to those obtained by other chromatographic methods.

In the Basic Protocol, gas-liquid chromatography (GLC) using an open tubular wall, coated, fused silica column with nonpolar liquid phase is described. In Alternate Protocol 1, reversed-phase HPLC (RP-HPLC) is applied for separation and quantification of cholesterol. In Alternate Protocol 2, enzymatic measurement is applied for determination of cholesterol.

MEASUREMENT OF CHOLESTEROL BY GAS CHROMATOGRAPHY

BASIC PROTOCOL

Prior to instrumental analysis, cholesterol is extracted from the food sample (*UNIT D1.1*) and saponified. The amount of the food sample depends on the cholesterol content; a sample containing ~1 mg of cholesterol is required for lipid extraction. Saponification hydrolyzes the carboxyl group attached to carbon in cholesterol and yields only free cholesterol in unsaponifiable matter. The unsaponifiable matter can be extracted by *n*-hexane, diethyl ether, or petroleum ether. Determination of cholesterol by GLC requires derivatization of the polar hydroxyl group of cholesterol to a nonpolar group such as trifluoroacetyl, acetyl, or TMS ether. This conversion of cholesterol allows high resolution of cholesterol from other sterol analogs by GLC. In the present protocol, unsaponifiable matter is extracted using *n*-hexane, and TMS ether derivatization of cholesterol is performed. This protocol requires prior knowledge of GLC methodologies and adherence to instructions from the instrument's manufacturer.

Materials

Food sample
5α-cholestane solution (internal standard; see recipe)
Methanol
Chloroform

Contributed by Toshiaki Ohshima
Current Protocols in Food Analytical Chemistry (2001) D1.3.1-D1.3.14

0.88% (w/v) KCl solution
Anhydrous sodium sulfate
0.5 M ethanolic potassium hydroxide solution (see recipe)
n-Hexane
Nitrogen gas
Pyridine
Hexamethyldisilazane (HMDS)
Trimethylchlorosilane imidazole (TMCS)
Cholesterol solutions (standard; see recipe)

Homogenizer with high-speed rotary blades
Vacuum filtration device
Whatman no. 1 filter paper
Separatory funnel
50-ml glass, tapered, round-bottom flask
Rotary evaporator
Condenser
60°C water bath
5-ml screw-cap glass vials
Gas chromatograph (GC) with:
  Flame ionization detector
  Open tubular-wall column (0.25-mm i.d. × 30-m) coated with OV-1 equivalent liquid phase (0.25-μm film thickness)
  Helium as a carrier gas
  Digital integrator

NOTE: All organic solvents should be reagent grade. HMDS and TMCS should be kept in a desiccator.

Extract total lipids

1. To an aliquot of food sample containing ~1 mg cholesterol, add 1 ml working 5α-cholestane solution (internal standard) followed by 10 vol methanol and homogenize for 5 min.

   Semi-solid and pasty samples must be sufficiently homogenized. Solid samples should be crushed and quantitatively passed through a chemical sieve with a <0.2-mm mesh.

2. Add 20 vol chloroform and homogenize again for 3 min.
3. Vacuum filter the homogenate through a Whatman no.1 filter paper and collect the filtrate fraction.
4. Add 10 vol methanol and 20 vol chloroform to the residue cake and homogenize 3 min.
5. Filter the homogenate as in step 3 and pool the second filtrate with the first.
6. Add ¼ vol 0.88% KCl solution and allow to separate into two layers in a separatory funnel.
7. Collect the lower (chloroform) layer containing total lipids and dry over anhydrous sodium sulfate.

Saponify total lipids

8. Transfer the chloroform solution of total lipids to a 50-ml glass, tapered, round-bottom flask. Remove chloroform under vacuum using a rotary evaporator at 30°C.

9. Add 20 ml of 0.5 M ethanolic potassium hydroxide solution.
10. Connect the flask to a condenser and reflux 30 min in a 60°C water bath to saponify the total lipids.

Extract unsaponifiable matter

11. Cool the refluxed solution to room temperature, remove the condenser, and transfer the saponified solution to a separatory funnel.
12. Add 60 ml water to dilute the alcohol, and subsequently extract the unsaponifiable matter three times with 20 ml *n*-hexane.

    When the amount of water added is small and the alcohol concentration in the saponified solution is high, fatty acid potassium salts may transfer into the ether layer along with unsaponifiable matter, including cholesterol. For quantitative recovery of cholesterol, as well as the complete separation of the fatty acid potassium salts from the cholesterol fraction, lowering the alcohol concentration by adding excess water prior to ether extraction is recommended. When the alcohol concentration is sufficiently low, the saponified solution forms easily.

13. Pool the *n*-hexane extracts and wash three times with 1 vol water. Dry over anhydrous sodium sulfate.

Prepare TMS ether derivative

14. Reduce the volume of *n*-hexane in vacuo with a rotary evaporator at 30°C and transfer the solution to a 5-ml screw-cap glass vial.
15. Dry the unsaponifiable matter under a nitrogen stream and add the following:

    0.5 ml pyridine
    0.2 ml HMDS
    0.1 ml TMCS.

16. Purge the head space using the nitrogen stream and screw the cap on tightly.
17. Incubate 30 min at ambient temperature to complete silylation.

Perform GLC

18. Remove excess pyridine under nitrogen at room temperature and add 1 ml *n*-hexane (final ~1 mg/ml cholesterol).
19. Inject in duplicate up to 2 µl *n*-hexane solution into the GC. Perform GLC using the following analytical conditions:

    Injection port and detector block: 330°C
    Column oven temperature program: 250° to 320°C at 4°C/min
    Column inlet helium pressure: 1.75 kg/cm^2 at 250°C
    Split ratio: 60:1.

    Under these analytical conditions, 5α-cholestane and the TMS ether derivative of cholesterol will be eluted at ~16 and 20 min, respectively.

20. Determine the peak areas of cholesterol and the internal standard with a digital integrator.

Run cholesterol standards

21. For each cholesterol standard concentration, prepare a mixture of 1 ml working 5α-cholestane solution (internal standard) and 1 ml working cholesterol solution (standard) in a 5-ml screw-cap glass vial.

22. Subject to silylation (steps 15 to 17) and analyze (steps 18 to 20).

Analyze data

23. Divide the area ratio of cholesterol in the samples by that of the internal standard to obtain a standard response ratio.

24. Plot the average response ratio against the ratio of cholesterol to internal standard in the standards.

ALTERNATE PROTOCOL 1

HPLC MEASUREMENT OF CHOLESTEROL

Prior to HPLC determination, total lipids, including cholesterol, are extracted from food and foodstuffs. As in the Basic Protocol, the amount of the food sample depends on the cholesterol content; a sample containing ~1 mg of cholesterol is required. Free fatty acids are completely removed from the cholesterol extract, and a benzoate ester derivative of cholesterol that has a specific UV absorbance at 230 nm is separated by RP-HPLC. This protocol allows accurate determination of cholesterol in food at levels as low as 10 ng benzoate derivative. This protocol requires prior knowledge of HPLC methodologies and adherence to instructions from the instrument's manufacturer. A typical result is shown in Figure D1.3.1.

Additional Materials *(also see Basic Protocol)*

HPLC-grade methanol
Saturated NaCl solution
Petroleum ether
Dry pyridine
Benzoyl chloride
0.1 M HCl
Diethyl ether
0.1 M Na_2CO_3
Cholesterol (>99% purity)

80°C water bath
60°C vacuum oven
High-performance liquid chromatograph (HPLC) with:
  Pump
  Sample injector
  Reversed-phase HPLC column: ODS column, 3.9-mm i.d. × 30-cm length, 10-µm particle size
  UV detector (230 nm)
  Digital integrator

NOTE: All organic solvents should be reagent grade. Diethyl ether should be kept at 4°C.

Extract and saponify lipids

1. Extract total lipids as described (see Basic Protocol, steps 1 to 7), but use HPLC-grade methanol and do not include 5α-cholestane as an internal standard.

2. Saponify lipids as described (see Basic Protocol, steps 8 to 10).

Remove free fatty acids

3. Add 10 ml *n*-heptane, reflux for another 2 to 3 min, and cool to room temperature.

4. Quantitatively transfer the refluxed solution to a separatory funnel and add 5 ml saturated NaCl solution.

5. Extract the aqueous phase twice with 10 ml petroleum ether.
6. Combine petroleum ether extracts and wash with 5 vol water. Dry over anhydrous sodium sulfate.

   When the amount of water added is small and the alcohol concentration in the saponified solution is high, fatty acid potassium salts may transfer into the ether layer along with unsaponifiable matter, including cholesterol. For quantitative recovery of cholesterol, as well as the complete separation of the fatty acid potassium salts from the cholesterol fraction, lowering the alcohol concentration by adding excess water prior to ether extraction is recommended. When the alcohol concentration is sufficiently low, the saponified solution forms easily.

7. Transfer the petroleum ether solution to a 50-ml glass, tapered, round-bottom flask and evaporate in vacuo using a rotary evaporator at 30°C.

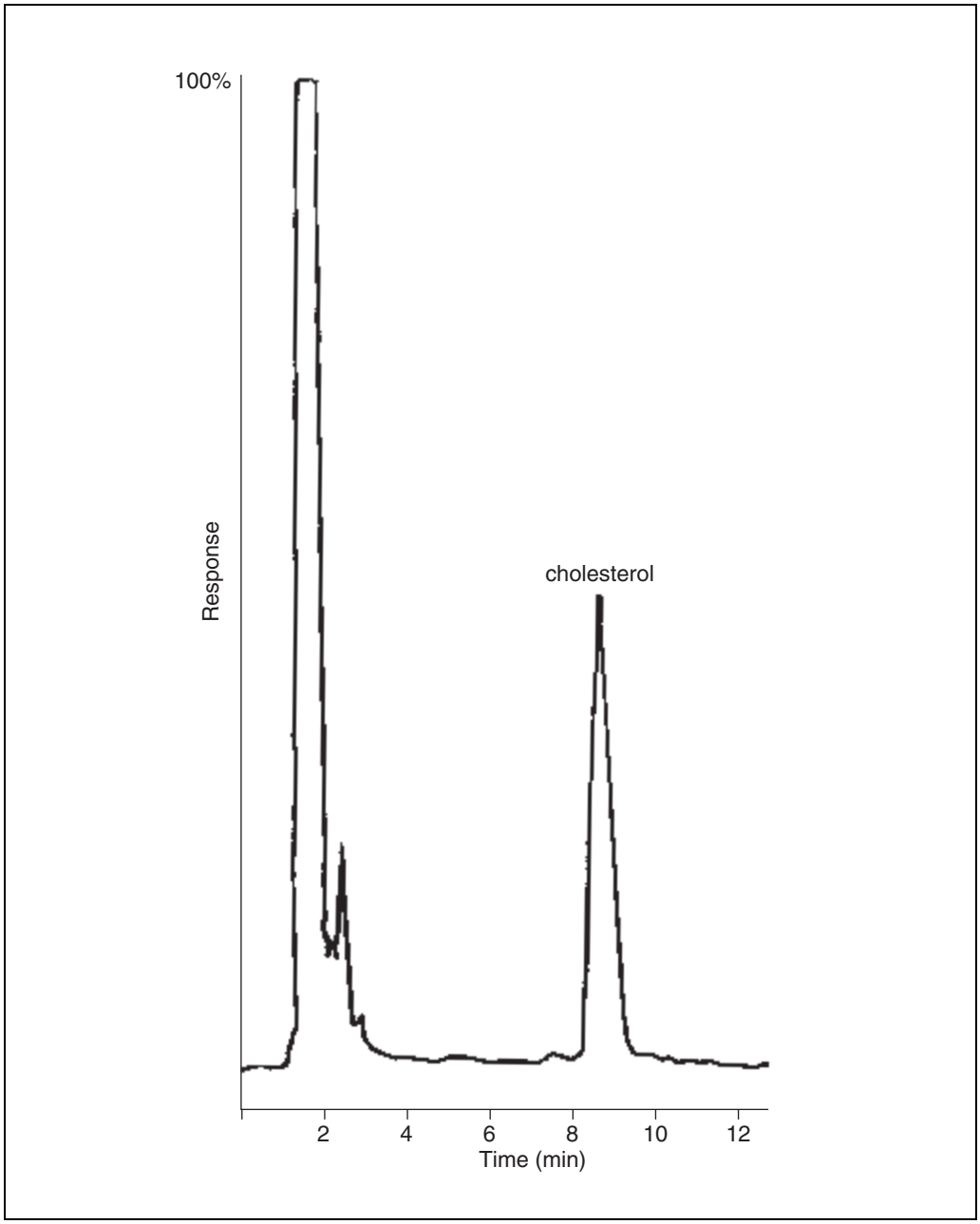

Figure D1.3.1 A typical HPLC chromatogram of cholesterol benzoate derived from dried egg yolk.

Prepare benzoate ester derivative

8. Transfer the solution to a 5-ml screw-cap glass vial.
9. Add 4 ml dry pyridine and 0.2 ml benzoyl chloride and stir for 5 min at room temperature.
10. Incubate 20 min in an 80°C water bath.
11. Pour the reaction mixture into a separatory funnnel, and add 50 ml of 0.1 M HCl and 50 ml diethyl ether to extract cholesterol benzoate.
12. Wash diethyl ether extract with 50 ml of 0.1 M HCl to remove excess benzoyl chloride.
13. Wash with 50 ml water.
14. Wash with 50 ml of 0.1 M Na_2CO_3 to neutralize the extract.
15. Wash again with water to remove excess carbonate.
16. Transfer to a beaker and evaporate to dryness under a nitrogen stream. Place the beaker in a vacuum oven at 60°C and dissolve the dried extract in 1 ml chloroform.

Prepare standards

17. Convert 100 mg cholesterol to benzoate derivative as above (steps 8 to 16).

    Commercially available authentic cholesterol usually absorbs moisture and exists as a monohydrate. Drying the authentic cholesterol at 80°C for 2 hr prior to use is recommended.

18. Prepare at least three different concentrations (~1 mg/ml) in duplicate.

Perform RP-HPLC

19. Inject 20 µl sample and standards into the HPLC and analyze using the following analytical conditions:

    HPLC column: ODS, 3.9-mm i.d. × 30-cm length, 10-µm particle size
    Mobile phase: methanol at 2 ml/min
    Detector: UV at 230 nm.

20. Prepare a calibration curve by plotting average detector response (peak area) of the standards versus cholesterol concentration.
21. Use the peak area of the sample and the calibration curve to determine the amount of cholesterol in the sample.

ALTERNATE PROTOCOL 2

ENZYMATIC MEASUREMENT OF CHOLESTEROL

Test combination kits for enzymatic determination of cholesterol in food are now commercially available. For the determination of total cholesterol, esterified cholesterol is hydrolyzed to free cholesterol and fatty acid under mild alkaline conditions. Cholesterol oxidase oxidizes free cholesterol to cholest-4-en-3-one to generate hydrogen peroxide, which further oxidizes methanol to formaldehyde. Formaldehyde then reacts with acetyl acetone in the presence of NH_4^+ ions to form yellow lutidine dye, which is subsequently determined spectrophotometrically.

Materials

Food sample
Sea sand
1.0 M methanolic potassium hydroxide solution (see recipe)
Isopropanol
Test combination F-kit for cholesterol determination (R-Biopharm), containing:
- Bottle 1:220,000 U catalase in 95 ml ammonium phosphate buffer, pH 7.0, and 2.6 M methanol (store up to 3 months at 4°C)
- Bottle 2: 0.05 M acetylacetone/0.3 M methanol (store up to 3 months at 4°C)
- Bottle 3: 12 U cholesterol oxidase suspension in a 0.8-ml volume (store up to 1 yr at 4°C)
- Bottle 4: 1.00 mg/ml cholesterol standard in isopropanol (store up to 3 months at 4°C)

50-ml volumetric flask
60°C and 37° to 40°C water baths
Reflux condenser
Whatman no. 5A filter paper
Brown glass bottle
10-ml glass test tubes
Glass cuvette with 1-cm light path
Spectrophotometer

NOTE: All organic solvents should be reagent grade.

Prepare sample

1. Accurately weigh ~1 g food sample into a 50-ml volumetric flask and add 1 g sea sand to complete saponification (step 3).

   Semi-solid and pasty samples must be sufficiently homogenized. Solid samples should be crushed and quantitatively passed through a chemical sieve with a <0.2-mm mesh. If the sample solution includes colorants, it should be treated with activated charcoal (e.g., with Clarocarbon F from Merck) at 5% of the sample weight.

2. Add 20 ml freshly prepared 1.0 M methanolic potassium hydroxide solution and 10 ml isopropanol.

3. Heat in a water bath at 60°C under a reflux condenser for 30 min while stirring with a magnetic stirrer.

4. Allow the turbid solution to cool. Remove the magnetic stir bar and bring to 50 ml with isopropanol at room temperature.

5. Mix the fluid and filter through Whatman no. 5A filter paper. Retain the clear sample solution for the assay.

Perform assay

6. Mix three parts catalase (bottle 1) with two parts acetylacetone/methanol (bottle 2) in a brown glass bottle. Allow to stand at room temperature 1 hr before use.

   This solution can be prepared in advance and stored up to 3 months at 4°C. Again, it should be allowed to stand at room temperature for 1 hr before use.

7. Prepare a sample blank (without cholesterol oxidase) by pipetting 5 ml catalase/acetylacetone/methanol solution and 0.4 ml sample solution into a 10-ml glass test tube.

8. Prepare the test sample by transferring 2.5 ml from the sample blank to a new tube and adding 0.02 ml cholesterol oxidase from bottle 3.

9. Mix thoroughly, cover the tubes, and incubate 60 min in a 37° to 40°C water bath.
10. Allow to cool to room temperature. Read absorbance of the sample blank and then the test sample in the same 1-cm glass cuvette against air at 405 nm.
11. Subtract absorbance of the blank from the absorbance of the sample. Determine concentration using the simplified equation c (g/liter) = $0.711 \times \Delta A_{405}$.

This equation is derived from the equation:

$$c = \left(\frac{V \times \text{mol. wt.} \times \text{DF}}{\varepsilon \times d \times v \times 100 \text{ mmol/mol}} \right) \times \Delta A_{405}$$

where V is final volume (5.4 ml), v is sample volume (0.4 ml), mol. wt. is the molecular weight of the substance to be assayed (386.64 g/mol), DF is the dilution factor (2.52/2.5 = 1.008), d is the light path (1 cm), and ε is the extinction coefficient of lutidine dye at 405 nm (7.4 liter/mmol/cm).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

5α-Cholestane solution

Prepare a 1.0 mg/ml 5α-cholestane (>99% pure) stock standard solution in *n*-hexane. Store up to 3 months at 4°C. Prepare a working solution by diluting the stock solution to 0.2 mg/ml in dimethylformamide (DMF). Prepare fresh.

Cholesterol solutions

Prepare a 1.0 mg/ml cholesterol (>99% pure) stock standard solution in dimethylformamide (DMF). Store up to 1 week at 4°C. Prepare working solutions by diluting the stock solution with DMF to obtain concentrations ranging between 0.05 and 0.5 mg/ml. Prepare fresh.

Commercially available authentic cholesterol usually absorbs moisture and exists as monohydrate. Drying the authentic cholesterol at 80°C for 2 hr prior to use is recommended.

Ethanolic potassium hydroxide solution, 0.5 M

Dissolve 5.6 g KOH in 2 ml water
Add 100 ml ethanol
Store up to 1 week at 4°C

Methanolic potassium hydroxide solution, 1.0 M

Dissolve 11.2 g KOH in 2 ml water
Add 100 ml methanol
Prepare fresh

COMMENTARY

Background Information

The TMS ether derivatives of sterols (including cholesterol) and their oxidation products in foods are well resolved by GLC. The TMS ether derivatives are eluted in the following order: cholesterol, campesterol, stigmasterol, and β-sitosterol.

Detection of cholesterol in extracted total lipids by HPLC is usually based on absorbance of short-wavelength UV light (200 to 210 nm; Duncan et al., 1979; Carrol and Rudel, 1981). Most organic compounds absorb UV light in this area of the spectrum, and therefore detection by UV light at ~205 nm is not specific to cholesterol itself. Thus, the Basic Protocol provides a more accurate determination of cholesterol in food matrix. Triglycerides consisting of three molecules of fatty acids are a predominant

proportion of most foods. Cholesterol is usually a small proportion compared to triglycerides. In the HPLC analysis of cholesterol as introduced in the present protocol, however, residual fatty acids in the cholesterol extract interfere with cholesterol detection and determination; fatty acids are co-eluted with cholesterol benzoate under the present HPLC conditions and saturate the UV absorbance at 230 nm. Complete removal of free fatty acids from the ether extract by sufficient saponification of the free fatty acids to corresponding potassium salts avoids this problem (Newkirk and Sheppard, 1981).

Although sterols exist as cholesterol in most food and food products including meat, fish, fats and oils, and milk (Kushiro et al., 1980), certain shellfish contain six other analogs of sterol, including 22-*trans*-24-norcholesta-5,22-diene-3b-ol, 22-dehydrocholesterol, brassicesterol, campesterol, 24-methylenecholesterol, and β-sitosterol. Because the relative activities of cholesterol oxidase to stigmasterol and β-sitosterol are 34% and 59%, respectively (while that to cholesterol is defined as 100%), overestimation of cholesterol content in shellfish and its products usually occurs during the enzymatic determination described in Alternate Protocol 2. Therefore, although enzymatic determinations of cholesterol in food and foodstuffs other than shellfish should be reliable, those in shellfish may be misread.

Several methodologies are known as effective tools for the determination of cholesterol in food and foodstuffs, e.g., colorimetry using the Libermann-Burchard reaction (Naito and David, 1984), Iatroscan thin-layer chromatography/flame ionization detection (Indrasena et al., 1991), enzymatic determination (Shen et al., 1982), GC determination (Karkalas et al.,

Table D1.3.1 Sample Dilution for Enzymatic Measurement of Cholesterol

Estimated amount of cholesterol per liter	Ratio of sample to isopropanol (v/v)	Dilution factor
<0.4 g	None	1
0.4-4 g	1:9	10
4-40 g	1:99	100

Table D1.3.2 Troubleshooting

Problem	Possible cause	Solution
For GC		
No peaks	Decomposition of TMS ether derivative due to moisture	Prepare derivative again under dry conditions
Large tailing peak at solvent front	Interference of residual pyridine in sample solution	Evaporate pyridine under a nitrogen stream
For HPLC		
Unexpected large peak of cholesterol	Free fatty acid remaining in ether extract	Add excess water and lower alcohol concentration during ether extraction
Many unknown peaks	Presence of oxidized cholesterol analogs	Perform GC measurement as recommended
For enzymatic determination		
Underestimation of cholesterol	Stabilizer in commercially available methanolic KOH solution may inhibit cholesterol oxidase	Prepare fresh solution in the laboratory
Too low optical density	Too low concentration in sample	Prepare sample: boil under reflux with KOH, extract with 1:1 ether/petroleum ether, evaporate organic phase, and dissolve residue in isopropanol

1982), HPLC determination (Osada et al., 1999), and HPLC/MS (Redden and Huang, 1991). In general, enzymatic determination is superior to colorimetry to obtain true cholesterol content. When food such as shellfish contains sterols other than cholesterol, the GC determination is the most adequate method. Although GC/MS also accomplishes good separation between and identification of all sterol analogs, the instrument is too expensive to use for routine analyses of cholesterol.

Critical Parameters

HPLC and GC measurement

Ethanolic potassium hydroxide irritates skin. Laboratory glasses should be worn during preparation to protect the eyes.

When pyridine has a pale yellow color, it should be distilled in glass prior to use for the derivatization of cholesterol benzoate or for TMS ether. The redistilled pyridine should be kept in a desiccator stored in the dark until used.

GC measurement

The TMS ether derivative should be kept in a desiccator until analyzed because it is very susceptible to moisture and hydrolyzes easily. Excess amounts of pyridine usually result in a large and tailing peak on a chromatogram and interfere with the baseline separation of the cholesterol peak. It is therefore recommended to evaporate pyridine under a nitrogen stream and to dissolve the residue in *n*-hexane. Excess silylating agents usually produce a white residue in the resulting *n*-hexane solution. Microfiltration prior to GC injection is recommended.

Table D1.3.3 Cholesterol Contents in Several Foods Determined by GC Measurement[a]

Food	Cholesterol (mg/100 g sample)	Food	Cholesterol (mg/100 g sample)
Fish, roe, and shellfish:		*Fats and oils:*	
Salmon	73	Lard	109
Jack mackerel	71	Butter	210
Mackerel	70	Margarine	0-2
Sea bass	49	Head	123
Sea bream	82		
Flounder	61	*Dairy:*	
Tuna	46	Milk	11
Rainbow trout	70	Yogurt	11
Salmon, roe	400	Natural cheese	69-75
Herring, roe	261	Ice cream (low fat)	26
Cod, roe	295		
Scallop	40	*Meats:*	
Squid	312	Beef, rump	76
Prawn	175-228	Chicken, breast	131
Octopus	66	Pork, rump	84
Scallop	100	Mutton	93
Abalone	91	Turkey	72
Sea urchin	219	Duck	76
		Rabbit	96
Eggs:			
Whole egg	428	*Others:*	
Egg yolk	1310	Mayonnaise	190
		Dressing	145

[a]From 4th Revision of Table of Japanese Food Standard Ingredients, Resources Council, Science and Technology Agency, Japan.

Table D1.3.4 Comparison of Cholesterol Contents of Poultry Meat and Cheese Determined by GC and Enzymatic Measurements[a]

Sample	Cholesterol (mg/100 g sample)	
	GC method	Enzymatic method
Chicken (raw):		
Skin	128	130
Leg	90	91
Wing	98	97
Light meat	67	70
Dark meat	107	109
Chicken (cooked):		
Skin	73	78
Leg	120	122
Wing	136	140
Light meat	80	80
Dark meat	92	93
Turkey (cooked):		
Light meat	82	79
Dark meat	89	84
Cheese:		
Edam	59	61
Double Gloucester	83	83

[a]Values are from Karkalas et al. (1982). Reprinted with permission from Blackwell Science Ltd.

Table D1.3.5 Substrate Specificity of Cholesterol Oxidase[a]

Sterols	Relative activity
Androsterone	0
Diosgenin	0
Testosterone	0
Estradiol	10
Stigmasterol	34
Dehydroepiandrosterone	37
β-Sitosterol	59
Cholesterol	100
β-Cholesterol	110
Pregnenolone	127

[a]Values are relative activity toward cholesterol and are from Kushiro et al. (1980). Reprinted with permission.

Table D1.3.6 Comparison of Cholesterol Contents of Shellfish Determined by GC and Enzymatic Measurements[a]

Sample	Cholesterol (mg/100 g sample)	
	GC method	Enzymatic method
Shijimi	125	147
Ark shell, *Scapharca broughtonii*	78	100
Short-neck clam, *Ruditapes phillipinarum*	76	95
Hard clam, *Meretrix lusoria*	69	89
Pacific oyster, *Crassostrea gigas*	76	107

[a]Values from Kushiro et al. (1980). Reprinted with permission.

HPLC measurement

Methanol used for the HPLC measurement should be of HPLC grade with low absorbance at UV wavelengths below 230 nm. Dried-processed foods usually include a relatively large amount of oxidized cholesterol analogs (Smith, 1996). The benzoate derivatives of these oxides do not separate from cholesterol benzoate by HPLC. Therefore, the GC measurement is more suitable for the determination of cholesterol in dried products with a high level of the oxidized cholesterol.

Enzymatic measurement

The potassium hydroxide solution should be prepared fresh before use, because commercially available methanolic potassium hydroxide solution usually contains a stabilizer that may inhibit cholesterol oxidase. The amount of cholesterol present in the test tube should be between 8 and 160 μg under the presented conditions to measure a sufficient difference in absorbance. The sample solution should be diluted as shown in Table D1.3.1.

Troubleshooting

Various problems and possible causes and solutions are shown in Table D1.3.2.

Anticipated Results

United States regulations on nutrition labeling of foods require that cholesterol content be given and that it be analyzed by GC measurement as shown in the AOAC method, which uses a packed column (Lewis et al., 1996; AOAC, 1990). The reference value, which is a set of recommended nutrient intake levels of cholesterol, is defined as 300 mg. However, the Codex guideline does not request labeling of cholesterol. Cholesterol contents in some foods and foodstuffs determined by GC measurement are summarized in Table D1.3.3.

As shown in Table D1.3.4, there is good agreement between the cholesterol contents of poultry meat and cheese determined by GC and those measured by the enzymatic method.

Cholesterol oxidase shows relatively wide substrate specificity to 3β-sterol analogs as shown in Table D1.3.5 As discussed above, cholesterol contents of certain shellfish, which contain a relatively large amount of 3β-sterol analogs, may be unreliable when determined by the enzymatic measurement as compared to results found using GC measurement, as shown in Table D1.3.6 (Kushiro et al., 1980).

A typical HPLC chromatogram of cholesterol benzoate obtained from dried egg yolk is shown in Figure D1.3.1.

Time Considerations

For GLC and HPLC analysis, the extraction of total lipids from food requires overnight separation of the organic layer. If the sample volume is small, however, centrifugation of the extract may shorten the sample preparation time. Cholesterol derivatization requires <2 hr depending on the number of samples. The peak of TMS ether and cholesterol benzoate derivatives will be eluted within 10 min after the injection of the sample.

Preparation of the sample for enzymatic measurement requires <30 min. One measurement requires <70 min.

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Full identification of isolated sterols from commercially consumable fats performed by GC/MS, and quantitative estimation of cholesterol content by capillary GC with flame ionization detection.

Caboni, M.F. and Rodriguez-Estrada, M.T. 1997. High-performance liquid chromatography coupled to evaporative light scattering detection in lipid analysis: Some application. *Seminars in Food Analysis* 2:159-169.

An evaporative light scattering detector was coupled with a UV spectrophotometer, and was applied to HPLC for the quantitative determination of cholesterol oxides in edible oils and fats.

Dutta, P.C., Caboni, M.F., Diczfalusy, U., Dionisi, F., and Dzeletovic, S. 1999. Measurements of cholesterol oxides in foods: Results of an interlaboratory comparison study. *Spec. Publ. R. Soc. Chem.* 240:309-315.

Study looking for good universal method for determination of eight analogs of cholesterol oxides in egg and milk powders. The results indicate difficulty in working with various food samples such as mixed diets.

Fetouris, D.J., Botsoglou, N.A., Psomas, I.E., and Mantis, A.I. 1998. Rapid determination of cholesterol in milk and milk products by direct saponification and capillary gas chromatography. *J. Dairy Sci*. 81:2833-2840.

For determination of cholesterol in milk and milk products, samples are saponified without lipid extraction in capped tubes with 0.5 M methanolic potassium hydroxide solution by heating at 80°C.

Labovics, V.K., Antal, M., and Gaal, O. 1996. Enzymatic determination of cholesterol. *J. Sci. Food Agric*. 71:22-26.

By separation of cholesterol oxides from cholesterol by TLC, it became possible to determine small amounts of cholesterol oxides by the enzymatic method even in the presence of a quantity of cholesterol.

Lopez-Hernandez, J., Gonzales-Castro, M.J., and Pineiro-Sotelo, M. 1999. Determination of sterols in sea urchin gonads by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. Sci.* 37:237-239.

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Manzi, P., Panfili, G., and Pizzoferrato, L. 1996. Normal and reversed-phase HPLC for more complete evaluation of tocopherols, retinols, carotenes and sterols in dairy products. *Chromatographia* 43:89-93.

A reversed-phase HPLC method was used for determination of cholesterol in dairy products. It evaluated that a more complete evaluation of cholesterol contents in dairy products is necessary when the vegetables and fruits are present.

Nielsen, H. 2000. Application of chemical methods to the determination of egg yolk. *Lebens.Wiss. Technol.* 33:151-154.

The adaptation of methods for lipid extraction and quantification by colorimetric determination of either ester or cholesterol in egg yolk with some contamination of egg white is described. Results are compared with those obtained by a conventional enzymatic determination.

Pasin, G., Smith, G.M., and O'Mahony, M. 1998. Rapid determination of total cholesterol in egg yolk using commercial diagnostic cholesterol reagent. *Food Chem.* 61:255-259.

By using a commercial diagnostic cholesterol reagent (enzymatic method) and gas chromatography, the cholesterol contents of four different egg yolk preparations (including whole egg powder, fresh, frozen, and dried egg yolk) were determined and compared.

Prygonski, K., Jelen, H., and Wasowicz, E. 2000. Determination of cholesterol oxidation products in milk powder and infant formulas by gas chromatography and mass spectrometry. *Nahrung* 44:122-125.

A method for determination of cholesterol oxidation products in milk powder and infant formulas is presented. Provides useful information on resolution of cholesterol oxidizing products from unoxidized cholesterol on a GC capillary column.

Toivo, J., Piironen, V., Kalo, P., and Varo, P. 1998. Gas chromatographic determination of major sterols in edible oils and fats using solid-phase extraction in sample preparation. *Chromatographia* 48:745-750.

A capillary gas chromatographic method is described for determination of major phytosterols and cholesterol in edible oils and fats. To extract the unsaponifiable matter and for sample cleanup, solid-phase extraction with C18 absorbent was used.

Contributed by Toshiaki Ohshima
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Oil Quality Indices

This unit defines three different tests that are used to evaluate lipid systems. The first two, i.e., iodine value (IV; see Basic Protocol 1) and saponification value (SV; see Basic Protocol 2), are used to determine the level of unsaturation and the relative size (chain length) of the fatty acids in the system, respectively. The free fatty acid (FFA) analysis (see Basic Protocol 3) is self-explanatory. Each of these analyses provides a specific set of information about the lipid system. The IV and SV provide relative information; this means that the data obtained are compared to the same data from other, defined lipid systems. In mixed triacylglyceride systems there is no absolute IV that indicates the exact number of double bonds or SV that indicates the exact chain length. The data from the FFA analysis is an absolute value; however, the meaning of the value is not absolute. As a quality indicator, ranges of FFA content are used and the amount that can be tolerated is product and/or process dependent.

DETERMINATION OF IODINE VALUE

BASIC PROTOCOL 1

The iodine value (IV) is used to determine the level of unsaturation in a fat/oil system. It is expressed as the number of grams of iodine that add to/react with 100 g of sample. The traditional iodine value method using the Wijs reagent requires carbon tetrachloride ($CC1_4$). For safety reasons, $CC1_4$ is no longer considered to be an acceptable chemical and it is not readily available for purchase, and if offered it is extremely expensive. Therefore the traditional method has been modified to a more "human-friendly" system which uses cyclohexane.

CAUTION: Wijs solution is hazardous and should be handled with gloves in a fume hood.

Materials

Fat or oil sample to be analyzed
Sodium sulfate (anhydrous; optional)
Cyclohexane
Wijs solution, in glacial acetic acid (Fisher Scientific; store in the dark at 25° to 30°C and keep tightly sealed)
Potassium iodide (KI) solution (see recipe)
Sodium thiosulfate ($Na_2S_2O_3$) standard solution (see recipe)
Soluble starch solution (see recipe)

Whatman no. 1 or no. 4 filter paper
68° to 70°C water bath
500-ml Ehrlenmeyer flask with 24/40 ground-glass joint
50-ml buret

1. Filter the sample through Whatman no.1 or no. 4 filter paper.

   The filter paper will remove any solid debris and can handle small amounts of water; however, if unsure, use anhydrous sodium sulfate (Na_2SO_4) to remove the water. This can be done either by placing some Na_2SO_4 in the filter paper and slowly pouring the oil through—this would be most efficient—or by putting the Na_2SO_4 in the oil for several hours (with occasional swirling) and then filtering the oil. If sample is a fat (solid or semisolid), then one must melt it prior to filtering; be careful to not heat >10°C over its actual melting point. Once a clean dry sample is obtained, it needs to be mixed sufficiently to give a representative sample.

2. After filtering, place the sample in a beaker and place the beaker in a water bath set at 68° to 70°C until the sample reaches this temperature.

Contributed by Susan L. Cuppett
Current Protocols in Food Analytical Chemistry (2001) D1.4.1-D1.4.12

Table D1.4.1 Sample Weights to be Used for Determination of Iodine Values Expected Iodine Value

Expected iodine value	Sample weight in ±0.001 g
<5	3.000
5-20	1.000
21-50	0.400
51-100	0.200
101-150	0.230
151-200	0.100

3. Once at desired temperature, immediately weigh the required amount of sample to the nearest 0.001 g into a 500-ml Ehrlenmeyer flask with a ground glass 24/40 joint.

   Sample size is based on expected IV (Table D1.4.1). If unsure try 1.000 g and see what happens, then adjust to get repeatable titration data.

4. Add 20 ml cyclohexane and swirl to dissolve the sample. Add 25 ml of Wijs solution using a pipet (not a graduated cylinder) for best control of data. Seal the flask with a glass stopper and place in a cool (25° to 30°C), dark place for 1 or 2 hr depending on expected IV (time must be consistent across samples). Also prepare a blank using all ingredients except the lipid sample.

   For an expected IV of <150 hold the sample for 1 hr; if >150 then hold for 2 hr.

   Cyclohexane serves to solubilize the fat. It should be purchased new and dated; if the data become erratic and no other source can be found for the problem, new cyclohexane should be obtained. Wijs solution is an iodine/chlorine solution in acetic acid. It acts to provide iodine monochloride (in excess) which reacts with the double bond. Wijs solution is sensitive to temperature, moisture, and light, and must be stored as recommended above.

5. At the end of the hold period, add 20 ml of KI solution, then immediately add 100 ml of distilled water. Swirl lightly to mix, then promptly titrate, with vigorous shaking/stirring, with 0.1 N sodium thiosulfate using a 50-ml buret. Continue titrating until the yellow-brown color has almost disappeared, then add 1 to 2 ml soluble starch indicator and continue to titrate until the blue/brown color has just disappeared. Record the volume of sodium thiosulfate that was used.

   The water forces the fat/oil into the cyclohexane and the excess iodine monochloride moves into the water, where it is converted to I_2 and can be titrated with the water-soluble sodium thiosulfate. Potassium iodide solution acts to convert the excess iodine monochloride to free iodine (blue) which can be titrated to a colorless end point with sodium thiosulfate. Soluble starch aids in being able to see the free iodine and getting a repeatable end point. The normality of the sodium thiosulfate solution used is generally 0.1 N; however, this may change depending on the level of iodine monochloride that is to be titrated. Ideally any titration should use between 5 and 50 ml (1 buret volume) of solution—titrations outside of this range can produce unreliable data.

6. Calculate the IV (g iodine/100 g sample) using the following equation.

$$\text{IV} = \frac{\left(\text{ml } Na_2S_2O_3 \text{ for blank} - \text{ml } Na_2S_2O_3 \text{ for sample}\right) \times Na_2S_2O_3 \text{ normality} \times 12.69}{\text{sample weight (g)}}$$

As a reference, the iodine values of oleic, linoleic, and linolenic acids are 89.9, 181.0, and 273.5, respectively. Iodine values for free fatty acids are higher than for those that are part

of a glyceride. If the double bonds are conjugated, then the value obtained is not a true measure of unsaturation. If one suspects that conjugation exists, then the oil needs to be assayed for conjugated dienes and trienes (see UNIT D2.1).

DETERMINATION OF SAPONIFICATION VALUE

BASIC PROTOCOL 2

The saponification value is the amount of alkali required to saponify a defined amount of sample. It is expressed in mg potassium hydroxide (KOH) per g sample. The procedure involves the use of excess alcoholic KOH, which catalyzes the saponification/release of the free fatty acids from the glycerol backbone. The unreacted KOH is then back-titrated with standardized hydrochloric acid (HCl) using phenolphthalein as the indicator. The amount and normality of the HCl used for neutralization can then be used to calculate the saponification value. The saponification value provides evidence as to the relative chain lengths of the fatty acids in the system.

Materials

Fat or oil sample to be analyzed
Standardized hydrochloric acid (purchase 1 N solution from Fisher or VWR and dilute to the needed normality, e.g., 0.5 or 0.1 N)
Alcoholic potassium hydroxide (see recipe)
Phenolphthalein solution (see recipe)

250-ml Ehrlenmeyer flasks or flat-bottom boiling flasks with 24/40 ground glass joints (for use with boiling water bath)
250-ml round-bottom boiling flasks with 24/40 ground glass joints (for use with heating mantles)
Water bath *or* heating mantles and voltage regulators
Air condensers (65 cm long; for use with boiling water bath)
Water cooled condensers (65 cm long; for use with heating mantles)
50-ml buret

Additional reagents and equipment for filtering/clarifying fat or oil sample (as for determining IV; see Basic Protocol 1)

1. Set up a reflux system and determine appropriate reflux temperatures using a preliminary sample.

   This can be done with a water bath or with heating mantles. Each system requires flasks with a ground glass joint (24/40 joint) to connect to a condenser; exact equipment will vary depending of heating system (see below). The water bath has the advantage of requiring less electrical materials, i.e., heating mantles, power strip, and voltage regulators. However, the water bath has the potential of flasks being upset due to buoyancy in the water, resulting in possible sample loss.

   a. *For the water bath:* Use either Erlenmeyer flasks or flat-bottom boiling flasks (250 ml) with a ground glass 24/40 joints. Attach air condensers (65 cm long). Place in water bath (make sure the flask and condenser do not "float" or become unstable) set to a temperature, determined with a preliminary sample, that creates a gentle boiling in the flask (e.g. 75° to 80°C).

   b. *For the heating mantle system:* Use round-bottom boiling flasks (250 ml) with ground glass 24/40 joints connected to a water-cooled condensers (65 cm long). Connect mantles to a voltage regulator for temperature control. Set the voltage regulator so that a gentle boil is reached; determine actual setting with a preliminary sample.

      Multiple mantles can be controlled through one voltage regulator using a power strip.

In etiher setup, the sample should boil gently and the vapor ring of the reflux should only rise about halfway up the condenser—it must not go all the way up the condenser since this means the ethanol is being evaporated from the system and the samples will be lost.

2. Cleaned fat/oil of any debris and/or water as described in Basic Protocol 1, step 1.

3. Weigh sample into flask to the nearest 0.01 g.

   The weight needs to be such that the back-titration will be about half (50%) that of the blank. Usually the sample size is 4 to 5 g; try a preliminary sample to make sure.

4. Quantitatively add 50 ml alcoholic KOH using a 50-ml pipet. Use the same pipet for every sample. Do not use a graduated cylinder. Prepare a blank sample by adding alcoholic KOH alone to a flask.

5. Attach the condenser and apply heat. Allow the sample to reflux until the sample is totally saponified.

   This is usually 1 hr; however, if unsure, do some preliminary work to see how much time is required. Indicators of completeness of saponification include having a clear, homogenous sample. A practical method for determining the amount of time required to saponify a sample is to saponify several samples under identical conditions and at regular times (30, 45, 60, 75, 90, and 120 min), remove a sample, and titrate it as described below. When a repeatable back-titration amount is reached, then the first saponification time resulting in that volume should represent the amount of time for full saponification. Use the appearance of the sample as a guide for when to start subsampling, e.g., if the sample is still cloudy at 30 min, there is no point in testing it yet.

6. Once saponification is complete, remove from heat—in water baths take out of the bath; if using heating mantles, lower the mantle away from the flask, making sure the flask is being held in place with a clamp. In either system, allow the sample to cool while still attached to the condenser. While still slightly warm to the hand, gently wash down the condenser with a small volume (3 to 5 ml) of distilled water

   Using a wash bottle makes it possible to wash down the condenser on all sides while keeping the volume of water used low; the next best choice is to use a disposable pipet. Try not to use a beaker; it lacks the control needed to get a good wash down and uses too much water.

7. Once the sample has cooled, disconnect it from the condenser, add ~1 ml phenolphthalein solution and titrate with the standardized HCl until a pink color that stays for 15 to 30 sec is obtained, indicating the end point. Record the amount of HCl used.

   The normality of the standardized hydrochloric acid can be adjusted if needed to get reliable data. With accurate dilution, the standard solution should be accurate, but if the researcher wishes to do an easy check, it can be titrated against a purchased standardized NaOH solution using phenolpthalein as the indicator. The standardized NaOH can be bought from either Fisher or VWR at a variety of concentrations. Ideally any titration should use between 5 and 50 ml (1 buret volume) of solution—titrations outside of this range can produce unreliable data.

8. Use the following calculation to determine the saponification value:

$$\text{saponification value} = \frac{(\text{ml HCl for blank} - \text{ml HCl for sample}) \times \text{HCl normality} \times 56.1}{\text{sample weight (g)}}$$

where 56.1 is the molecular weight of KOH.

MEASUREMENT OF FREE FATTY ACIDS OR ACID VALUE

This procedure measures the amount of free acid groups existing in fat/oil systems. It can reflect total acidity (acid value; AV) or it can reflect the level of fatty acids that are free, i.e., not attached to a glycerol backbone. FFA is defined as the percentage by weight of free acid groups in the oil, while AV is defined as the weight (mg) of potassium hydroxide required to neutralize the free acid groups in oil. Fatty acids are usually the predominant source of free acid groups; however, acid phosphates, and, in some instances, amino acids can contribute to the level of acid groups available in the system. If the FFA level is only attributable to fatty acids, then the FFA value and AV can be converted using a conversion factor. However, the conversion factor is specific to single fatty acids. For oleic acid the conversion is made as follows: %FFA (oleic) × 1.99 = AV.

The procedure for measuring FFA in oil is relatively simple. Initially the oil needs to be clean, i.e., free of debris and sediment, and dry. This can usually be accomplished by filtering the oil. Once the sample is ready, one needs to have an idea of the level of FFA in the sample; this affects the amount of oil that is to be tested. A reference for sample size and amount of alcohol and normality of sodium hydroxide to be used is given below in Table D1.4.2. If unsure, take a "best guess" and after titrating the test sample, evaluate the data, and adjust sample size and/or normality until a repeatable analysis is obtained.

Materials

Fat or oil sample to be analyzed
Neutralized ethanol (see recipe), preheated to 60° to 65°C
Phenolphthalein solution (see recipe)
Standard sodium hydroxide (normality depending on sample size; see Table D1.4.2; solution can be purchased)
250-ml Ehrlenmeyer flask

Additional reagents and equipment for filtering/clarifying fat or oil sample (as for determining IV; see Basic Protocol 1)

1. Clean sample of debris and/or water as described in Basic Protocol 1, step 1.
2. Based on the expected FFA level, select (Table D1.4.2) sample size, amount of alcohol, and normality of sodium hydroxide to be used.
3. Weigh the sample (to 0.001 g) into a 250-ml Erlenmeyer flask.
4. Add the appropriate volume of neutralized alcohol (Table D1.4.2), preheated to 60° to 65°C, then add 1 ml of phenolphthalein solution. Swirl to make sure the system is well mixed.
5. Titrate immediately with sodium hydroxide solution (see Table D1.4.2 for appropriate normality). Swirl vigorously throughout the titration. Titrate to the same permanent

Table D1.4.2 Sample Weights and Alcohol Volumes for Determination of Free Fatty Acid Content in Fats and Oils

Expected FFA range (%)	Sample weight (g)	Amount of alcohol (ml)	Normality of sodium hydroxide
0.0-0.2	56.4 ± 0.20	50	0.10
0.2-1.0	28.2 ± 0.20	50	0.10
1.0-30.0	7.05 ± 0.05	75	0.25
30.0-50.0	7.05 ± 0.05	100	0.25 or 1.0
50.0-100.0	3.525 ± 0.001	100	1.0

pink color and intensity as was observed when preparing the neutralized ethanol (see Reagents and Solutions). Record the amount of sodium hydroxide solution used.

It is recommended that a 1 N solution be purchased and diluted to the needed normality. Ideally any titration should use between 5 and 50 ml (1 buret volume) of solution—titrations outside of this range can produce unreliable data.

6. Calculate results.

   FFA values are usually reported as oleic acid; however, in oils such as coconut and palm kernel, FFA is reported in terms of lauric acid or palmitic acid, respectively.

   For oleic acid calculate FFA as:

$$\text{FFA as \% oleic acid} = \frac{\text{ml NaOH} \times \text{NaOH normality} \times 28.2}{\text{weight of sample (g)}}$$

   If calculating based on lauric acid, 20.0 is substituted for the 28.2; for palmitic acid substitute 25.6. These are the equivalent weights of the fatty acid being calculated. FFA content calculation in these cases is determined based on the predominant fatty acid found in the sample. The calculation can also be based on the average molecular weight of the fatty acids found in the sample.

7. Calculate acid value as follows:

   FFA × 1.99 for oleic acid
   FFA × 2.81 for lauric acid
   FFA × 2.19 for palmitic acid.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alcoholic potassium hydroxide (KOH)

Put ~500 to 600 ml of 95% ethanol in a 1-liter beaker. Keep the ethanol on a stir plate with a moving stir bar then slowly add 40 g of KOH pellets—i.e., add some, allow to dissolve, then add some more, etc. Alkalis release large amounts of heat when dissolving, so make sure the beaker does not get too hot. Once all of the pellets are in solution, allow the solution to come to room temperature, then transfer to a 1-liter volumetric flask and bring to volume with 95% ethanol. Let stand overnight and then filter through Whatman no. 1 or no. 4 filter paper prior to use.

The solution should remain clear. It is best made fresh; if allowed to stand too long, it will turn brown and should not to be used. Be aware that KOH is strongly caustic and must be handled with gloves. It is also hygroscopic, so weighing should be done rapidly.

Neutralized ethanol

Titrate 95% ethanol with a few drops of standardized alkali in the presence of phenolphthalein indicator until a faint pink color is obtained that persists for 15 to 30 sec.

Make a note of the color and intensity of the endpoint; this should be the endpoint for the sample titrations as well.

Phenolphthalein solution

Weigh 1 g phenolphthalein and place the powder in a 100-ml volumetric flask containing ~50 ml of 95% ethanol. Stopper and shake vigorously for a few minutes, then add ~20 ml more ethanol and shake until the phenolphthalein is in solution. Bring to 100 ml and shake for homogeneity. Transfer to a glass bottle for storage.

The 95% ethanol can be made from 100% by proper dilution; however, make sure the 100% ethanol has not been stabilized, especially with pyridine, since pyridine has significant buffering capacity.

Potassium iodide (KI) solution

Dissolve 15 g KI in 100-ml distilled water in a volumetric flask, then transfer to a brown glass bottle (or wrap bottle in aluminum foil) and store in a cool, dark place.

KI is very light sensitive, and should not be used if it turns brown.

Soluble starch solution, 0.5%

Measure out ~150 ml of water and put it in a 500-ml beaker. Place the beaker onto a heat/stir plate. Add a stir bar and start stirring. Begin heating the water; once it gets very warm to the touch, slowly mix in 1 g of soluble starch. Take the solution to a gentle boil and continue boiling until all of the starch goes into solution, i.e., the solution becomes clear. Let cool and transfer to a 200-ml volumetric flask, then bring to volume. Transfer to a storage bottle. Store at 4°C, and check it each time it is to be used for signs of mold growth. If mold problems are suspected, discard and remake.

Sodium thiosulfate standard solution, 0.1 N

Purchase from Fisher or VWR or prepare as follows. (1) Weigh 24.82 g of $Na_2SO_3 \cdot 5H_2O$, transfer to a l-liter volumetric flask and take to volume with distilled water (to hasten solubilization, only add ~300 to 500 ml, shake until dissolved, then take to volume). (2) Standardize against potassium dichromate ($K_2Cr_2O_7$; exercise caution; this a strong oxidant and can damage skin). Initially dry the $K_2Cr_2O_7$ for 2 hr in an oven set at 100°C, cool in a desiccator, and accurately weigh (to four decimal points) between 0.2 and 0.23 g of $K_2Cr_2O_7$. Dissolve in 80 ml of distilled water, then add 2 g of potassium iodide (KI) and 8 ml of concentrated hydrochloric acid. Mix thoroughly, then titrate with the sodium thiosulfate solution until the brown color has just changed to yellowish-green. Add 1 ml of soluble starch solution (see recipe) and titrate until the blue color becomes light green. Record the amount of sodium thiosulfate used. The normality of the sodium thiosulfate solution can be calculated using the following equation.

$$\text{Normality of sodium thiosulfate} = \frac{\text{g } K_2Cr_20_7 \times 1000}{\text{ml } Na_2S0_3 \times 49.032}$$

COMMENTARY

Background Information

Iodine value (IV)

This analysis measures the unsaturation in a lipid system. It is based on the fact that halogens will react with aliphatic double bonds in a measurable way. Initially the sample is mixed with the Wijs solution, which contains excess iodine monochloride (ICl). The ICl binds to the double bond(s) as follows:

$$\underset{\text{(excess)}}{\text{ICl}} + \text{R-CH=CH-R} \rightarrow \text{R-CHI-CHCI-R} + \underset{\text{(remaining)}}{\text{ICl}}$$

The remaining ICl can then be measured after the addition of potassium iodide (KI). The KI reduces the excess ICl to I_2:

$$\text{ICl} + 2\text{KI} \rightarrow \text{KCl} + \text{KI} + \text{I}_2$$

which can be measured by titration with a standardized sodium thiosulfate solution.

Iodine values in pure systems (Table D1.4.3) have been reported, and it can be seen that within sets of triglycerides with fatty acids of about the same or with the same chain lengths but with increasing numbers of double bonds, there is an increase in IV. However, within a series of triglycerides with increasing chain lengths (from 10 to 24) with one double bond,

Table D1.4.3 Molecular Weights and Related Characteristics of Unsaturated Fatty Acids[a]

Fatty acid	No. C atoms	No. double bonds	Mol. wt.of acid	Mol. wt. of triglyceride	Saponification value of triglyceride	Iodine value of acid	Iodine value of triglyceride
Decenoic	10	1	170.24	548.8	306.7	149.1	138.77
Dodecenoic (lauroleic)	12	1	198.29	632.9	265.9	128.0	120.32
Tetradecenoic (myristoleic)	14	1	226.34	717.1	134.7	112.1	106.20
Hexadecenoic (palmitoleic)	16	1	254.39	801.2	210.1	99.78	95.04
Octadecenoic (oleic etc.)	18	1	282.44	885.4	190.1	89.87	86.01
Eicosenoic (gadoleic)	20	1	310.50	969.5	173.6	81.75	78.54
Docosenoic (erucic etc.)	22	1	338.55	1053.7	159.7	74.98	72.27
Tetracosenoic (selacholeic)	24	1	336.60	1137.9	147.9	69.24	66.93
Octadecadienoic (linoleic)	18	2	280.43	879.3	191.4	181.04	173.21
Hexadecatrienoic	16	3	250.36	789.2	213.3	304.17	289.48
Octadecatrienoic (linolenic etc.)	18	3	278.41	873.3	192.7	273.52	261.61
Octadecatetraenoic	18	4	276.40	867.3	194.1	367.35	351.21
Eicosatetraenoic (arachidonic etc.)	20	4	304.45	951.4	176.9	333.51	320.17
Eicosapentaenoic	20	5	302.43	945.3	178.1	419.67	402.79
Docosapentaenoic	22	5	330.49	1029.5	163.5	384.04	369.85
Docosahexaenoic	22	6	328.47	1023.5	164.4	463.68	446.42
Tetracosapentaenoic	24	5	358.54	1113.7	151.1	353.99	341.89
Tetracosahexaenoic	24	6	356.52	1107.6	152.0	427.20	412.52
Hexacosapentaenoic	26	5	386.59	1197.8	140.5	328.31	317.88
Hexacosahexaenoic	26	6	384.57	1191.8	141.2	396.04	383.38

[a] Adapted from Bailey (1945).

the IV decreases with increasing chain length. The IV is important when establishing basic information on an oil—for instance, a high IV could indicate less stability to oxidation. Table D1.4.4 shows the IV and fatty acid composition of some commonly used oils; another source of information on the IV of oilseeds, especially some of the tropical oils is the book edited by Rossell and Pritchard *Analysis of Oilseeds, Fats and Fatty Foods* (Rossell and Pritchard, 1991).

Traditionally, another use of IV has been to monitor the hydrogenation process and establish the hydrogenation end point relative to the finished product's functionality (solid fat index or SFI). A typical result from a hydrogenation of canola oil is shown in Table D1.4.5. As the level of hydrogenation increases, as evidenced by the loss of unsaturation (decreased IV), there is an increase in the SFI, i.e., the fat melts over a longer range of temperatures.

Saponification value (SV)

As indicated previously, this analysis provides a measure of the mean molecular weight of the fatty acids in a lipid system. It is based on the fact that saponification breaks ester bonds in a lipid system and since fatty acids are attached to the glycerol backbone with an ester bond, SV reflects the number of ester bonds per gram sample. The saponification value indicates the mean molecular weight of the sample's triacylglycerols and when divided by 3 gives the mean molecular weight of the constituent fatty acids. Therefore, the smaller the

Table D1.4.4 Properties and Composition of Selected Fats[a]

Fat	Iodine value	Saponification value	Fatty acid composition (wt %)					
			14:0	16:0	18:0	18:1	18:2	18:3
Corn	116-140	118-198		7	3	43	39	
Cottonseed	90-112	189-198	1.5	22	5	19	50	
Linseed	168-204	188-196		6	4	13-37	5-23	26-58
Olive	76-90	186-196	1.3	7-16	1.4-3.3	64.5-84.5	4-15	
Palm	35-61	195-205	0.6-2.4	32-45	4-6.3	38-53	6-12	
Peanut	84-102	188-195	0.5	6-11.4	3-6	42.3-61	13-33.5	
Rapeseed, regular	94-106	168-179	1.5	1-4.7	1-3.5	13-38	9.25-22	1-10
Safflower, regular	126-152	175-195		6.4-7.0	2.4-2.8	9.7-13.1	76.9-80.5	
Safflower, high oleic	90-100	175-195		4-8	4-8	74-79	11-19	
Sesame	104-116	187-193		7.2-7.7	7.2-7.7	35-46	35-48	
Soybean	117-140	189-195		2.3-10.6	2.4-6	23.5-30.8	49-51.5	2-10.5
Sunflower	112-143	186-194		3.5-6.5	1.3-3	14-43	44-68	

[a] Adapted from Fulmer (1990).

Table D1.4.5 Iodine Value (IV), Fatty Acid Composition, and Solid Fat Index (SFI) of Canola Oil[a]

IV	Fatty acid composition (%)						SFI at				
	C16: 0	C18: 0	C18: 1	C18: 2	C18: 3	Trans	10.0°C	21.1°C	26.7°C	33.3°C	40.0°C
115 (starting oil)	4.5	2	60	22	10	—	—	—	—	—	—
90	4.5	3	80	9	2	20	2.5	—	—	—	—
85	4.5	5	86	2	1	42	18	5	—	—	—
80	4.5	11	83	—	—	45	30	18	7	<1	—
75	4.5	14	80	—	—	50	50	31	25	11	4

[a] Adapted from Hui (1996).

saponification value, the longer the fatty acids on the glycerol backbone; conversely, a high value indicates shorter fatty acids. Generally, the method is used with triacylglycerides; however, this method can be useful for evaluating other lipid systems such as waxes.

The basic chemistry is as follows. The lipid system is mixed with excess alcoholic alkali (KOH) in the presence of heat; this breaks the ester bond releasing the fatty acids as salts (Fig. D1.4.1). The remaining alkali can then be measured by back-titration with a standardized acid solution.

Again, as with the IV, the saponification value provides some basic information about a lipid system. In a pure system (Table D1.4.3), with a series of triglycerides of increasing chain length, as the chain length increases there is a decrease in saponification value. With triglycerides of the same or close chain lengths, there is little difference in saponification value. Table D1.4.4 shows saponification values of some common oils, and again more information can be found in the book by Rossell and Pritchard (1991), as indicated above.

Free fatty acid (FFA)

This analysis measures the amount of free acid, and is used to reflect the level of FFA in the sample. Free fatty acids are significant for the quality of the oil because they increase the oil's susceptibility to oxidation, can contribute bitter/soapy flavors, and can cause a decrease in the oil's smoke and flash points.

Free fatty acids indicate the breakage of the ester bond between the fatty acid and the glycerol backbone, releasing the fatty acid (Fig. D1.4.2). The breakage of the ester bond can occur due to the presence of lipases, as found in damaged seeds, or as the result of hydrolytic

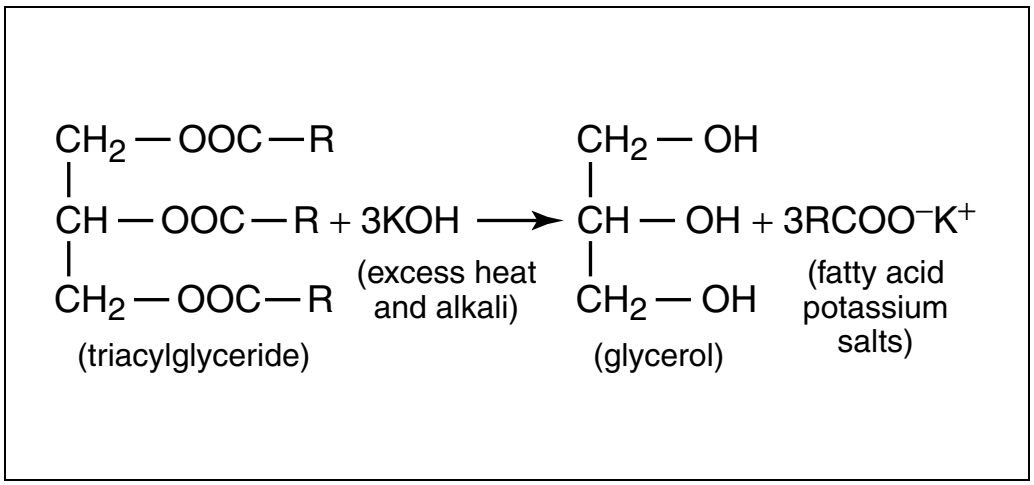

Figure D1.4.1 Saponification of a triglyceride by alkali.

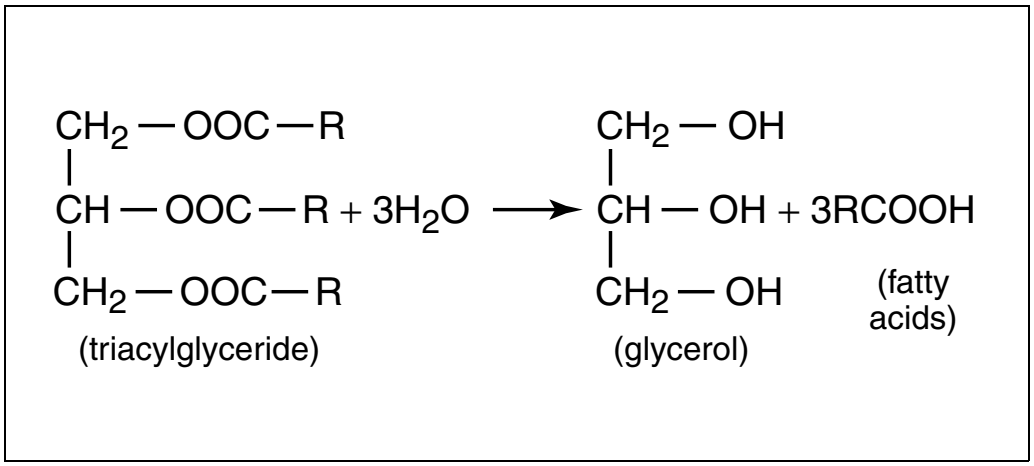

Figure D1.4.2 Hydrolysis of a triglyceride by lipase or heat to produce free fatty acids.

cleavage due to high heat and the presence of water, as in deep-fat frying. Both of these instances are of concern to food processors. When oils are refined, the final oil should contain a maximum of 0.05% FFA (based on oleic acid); most refined oils contain ≤0.03% FFA. In deep-fat frying, the FFA content of the oil is critical for maintaining final product quality and shelf life. In addition, as the FFA content increases, the oil's smoke and flash points drop, and without proper monitoring, the oil could produce excessive smoke and eventually ignite (flash) into flames in the processing arena. Ideally frying oil FFA content should not be greater than 2%.

Critical Parameters

Iodine value

Since this analysis is based on iodine, great care must be taken to protect the KI solution from light. In the presence of light the KI solution will turn brown and cannot be used. Even if stored in the dark, over time the KI solution will turn brown; therefore, before starting this analysis always check to make sure the KI solution is colorless. The other solution that has to be monitored is the soluble starch; even stored in the refrigerator, it will, over time, become moldy and unusable.

Saponification value

This analysis is a relatively simple procedure; however, it is critical that the saponification be complete prior to the final titration. Therefore, it is important to establish the amount of time needed to have a representative sample. See note in the text on how to know when the sample is totally saponified.

Free fatty acids

This is a simple procedure and if one follows the steps it will work. The crucial part is to not hold the neutralized alcohol at 65°C for a long

period of time (>30 min). As the alcohol evaporates, the alkali used to neutralize it concentrates, and this will change the endpoint.

Anticipated Results

Iodine value

The iodine value is reported as the number of grams of iodine that bind to 100 g of sample. As such, the IV can only be used as an index of unsaturation, especially in a mixed triacylglyceride system. This means that it can be compared to the same data from other mixed triacylglyceride systems, i.e., comparing to a competitor oil/fat. The other possible use is to reflect changes in the value based on "treatment" effects occurring in the system in question, e.g., to monitor hydrogenation.

Saponification value

Data collected from a mixed triacylglyceride system can only be used as a relative index of chain length. This means that it can be compared to the same data from other mixed triacylglyceride systems.

Free fatty acids

Data collected from a mixed triacylglyceride system, although a hard number, can only be used as a relative index of free fatty acids. This means that it can be compared to the same data from other mixed triacylglyceride systems, i.e., to a competitor oil/fat. The other use is to reflect changes in the value based on "treatment" effects occurring in the system in question. For instance in refining, FFA indicates the effectiveness of the alkali washing of the system; during storage FFA can indicate deterioration due to handling and/or presence of lipases. In deep-fat frying, knowledge of FFA content is critical to having a safe process and a storable product.

Time Considerations

Iodine value

For this analysis, the time needed is determined by the fact that all of the samples must be titrated after being held for either 1 or 2 hr, and this time should be consistent across the samples. With good planning, it is possible to increase productivity. For instance, it is possible to stagger samples so that they can be being titrated every few minutes. When preparing samples, each new one can be started a 3 to 5 min behind the previous one, so that at the end of the time for the first sample, there will be a progression of samples ready to titrate every few minutes. If sufficient time exists between the titrations, the analyst can start new samples. This system requires the full concentration of the analyst. Each titration should only take 2 to 3 min once the analyst has become comfortable with the method.

Saponification value

The amount of time required is based on the fact that the saponification will require a set amount of time, followed by a titration. The titration should only take 2 to 3 min once the analyst has become comfortable with the method. The primary restriction to doing a lot of samples is the amount of equipment available for the saponification step. Generally, using the heating mantles and a power strip, six setups can be maintained with ease. In a water bath, the number is controlled by the size of the bath, but again 6 to 8 should be possible.

Free fatty acids

This procedure is based on the fact that the neutralized alcohol has to be warm prior to titration, and care has to be taken to not evaporate the alcohol by allowing the warm solution to sit for extensive periods of time. However, once the analyst is comfortable with the method, it should be possible to generate samples quickly, since the titration should only take 2 to 5 min.

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American Oil Chemists' Society. 1993c. AOCS Official Method Cd 3-25. Saponification Value. Champaign, Ill.

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These references provide basic information defining good laboratory procedures, as well as valuable insight into the analyses described in this unit.

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Analysis of Tocopherols and Tocotrienols

Many techniques are used in the analysis of tocopherols and tocotrienols. In 1970s, the Association of Official Analytical Chemists' (AOAC) International *Official Methods of Analysis* presented several conventional analysis methods for α-tocopherol and α-tocopheryl acetate (Table D1.5.1). These methods are based on colorimetric or polarimetric analysis and thin-layer chromatography techniques. The problem with these methods is that many interfering compounds are not efficiently eliminated and have an impact upon the reliability of results. Also, a relatively large quantity of sample is required due to low sensitivity. Gas chromatography (GC) methods were developed to determine tocopherols and tocotrienols in the late 1980s. Several such methods have been described in the AOAC International *Official Methods of Analysis* and are listed in Table D1.5.2. The analytical sensitivity is significantly improved with the GC method compared with the colorimetric and polarimetric methods. However, because the boiling points of tocopherols and tocotrienols are high and relatively close together, GC separation of tocopherols and tocotrienols is difficult to achieve. To decrease their boiling points and avoid decomposition of tocopherols and tocotrienols at the high analysis temperature that must be applied in the GC method, a derivatization procedure is usually carried out during sample preparation. The derivatization reaction transforms the hydroxyl groups of tocopherols and tocotrienols to trimethylsilyl (TMS) forms, resulting in lower boiling points. However, the conditions of derivatization are very critical for obtaining high yields of derivatives, and high variability may occur between duplicates.

Currently, high-performance liquid chromatography (HPLC) methods have been widely used in the analysis of tocopherols and tocotrienols in food and nutrition areas. Each form of tocopherol and tocotrienol can be separated and quantified individually using HPLC with either a UV or fluorescence detector. The interferences are largely reduced after separation by HPLC. Therefore, the sensitivity and specificity of HPLC methods are much higher than those obtained with the colorimetric, polarimetric, and GC methods. Also, sample preparation in the HPLC methods is simpler and more efficiently duplicated than in the older methods. Many HPLC methods for the quantification of tocopherols and tocotrienols in various foods and biological samples have been reported. Method number 992.03 of the AOAC International *Official Methods of Analysis* provides an HPLC method to determine vitamin E in milk-based infant formula. It could probably be said that HPLC methods have become dominant in the analysis of tocopherols and tocotrienols. Therefore, the analytical protocols for tocopherols and tocotrienols in this unit are focused on HPLC methods. Normal and reversed-phase HPLC methods are discussed in the separation and quantification of tocopherols and tocotrienols (see Basic Protocol). Sample

Table D1.5.1 Conventional Analysis Methods of α-Tocopherol and α-Tocopheryl Acetate in the AOAC International *Official Methods of Analysis*

Method	Application
AOAC 971.30	α-Tocopherol and α-Tocopheryl Acetate in Foods and Feeds, Colorimetric Method (First Action 1971 and Final Action 1972)
AOAC 975.43	Identification of RRR- or *all-rac-alpha*-Tocopherol in Drugs and Food or Feed Supplements, Polarimetric Method (First Action 1975 and Final Action 1980)
AOAC 948.26	α-Tocopheryl Acetate (Supplemental) in Foods and Feeds, Colorimetric Method (Final Action 1980)

Contributed by Zhimin Xu

Current Protocols in Food Analytical Chemistry (2002) D1.5.1-D1.5.12

Table D1.5.2 GC Analysis Methods of α-Tocopherol and α-Tocopheryl Acetate in the AOAC International *Official Methods of Analysis*

Method	Application
AOAC 969.40	Vitamin E in Drugs, Gas Chromatographic Method (First Action 1969 and Final Action 1974)
AOAC 988.14	Tocopherol Isomers in Mixed Tocopherols Concentrate, Gas Chromatographic Method (First Action 1988)
AOAC 989.09	α-Tocopheryl Acetate in Supplemental Vitamin E Concentrates, Gas Chromatographic Method (First Action 1989)

preparation for various foods—such as refined oils (see Support Protocol 1), oil and fat products (see Support Protocol 2), meats (see Support Protocol 3), and cereals and nuts (see Support Protocol 4)—are also described.

BASIC PROTOCOL

HPLC ANALYSIS OF TOCOPHEROLS AND TOCOTRIENOLS

There are numerous papers that refer to the quantification of tocopherols and tocotrienols using HPLC methods. Normal-phase HPLC methods with a silica column as well as reversed-phase HPLC methods with a C18 column are commonly used. A silica normal-phase column is able to separate all eight tocopherols and tocotrienols in a typical chromatographic procedure. Because plant tissues possess most forms of tocopherol and tocotrienol, it is recommended that the normal-phase HPLC method be applied to food samples from plants. In the reversed-phase HPLC method, β- and γ-tocopherol and β- and γ-tocotrienol are not usually completely separated. This method can be used in animal tissues, which either lack or have reduced levels of β- and γ-tocopherol and β- and γ-tocotrienol. The resolution of the normal-phase HPLC method is higher than that of reversed-phase HPLC method; however, the reversed-phase HPLC column is more long-lasting than the normal-phase HPLC column (see Critical Parameters and Troubleshooting).

Materials

Mobile phase (all HPLC grade):
1:1:198 (v/v/v) ethyl acetate/acetic acid/hexane for normal phase
25:22:3 (v/v/v) methanol/acetonitrile/methylene chloride for reversed phase
Sample to be analyzed (e.g., see Support Protocols 1, 2, and 4 for normal phase; see Support Protocols 2 and 3 for reversed phase)
Standards: α-, γ-, and δ-tocopherols (Sigma-Aldrich) and tocotrienols (Shin and Godber, 1994)

High-performance liquid chromatograph (HPLC) with fluorescence detector (excitation 290 nm, emission 330 nm) or UV detector (295 nm)
Analytical column, 4.6 mm × 25 cm, 5-μm particle size:
silica (e.g., Supelcosil LC-Si, Supelco) for normal phase
C18 (e.g., Supelco Discovery C18) for reversed phase

1. Set up HPLC system according to manufacturer's instructions.
2. Flush system with mobile phase for 10 min at a flow rate of 1.5 ml/min.
3. Connect column and detector.
4. Equilibriate column with mobile phase at a flow rate of 1.5 ml/min until baseline is stable.

5. Prepare different concentrations of standard solution using mobile phase (generally, 0 to 10 μg/ml for α- and γ-tocopherols and tocotrienols and 0 to 5 μg/ml for other tocopherols and tocotrienols).
6. Inject standard solutions (usually 25 μl) and analyze using mobile phase at a flow rate of 1.5 ml/min for normal phase or 1.0 ml/min for reversed phase.
7. Convert injection quantities of standards to μg unit:

   Injection quantity of standard (μg) = concentration of standard (μg/ml) × injection volume (ml).
8. Create standard curve (peak area versus injection quantity).
9. Inject sample (25 to 100 μl) and analyze using the same conditions as in step 6.
10. Determine the quantity of the tocopherol or tocotrienol that corresponds to the peak area from the sample using the standard curve.
11. Calculate concentration in the raw sample according to the following equation:

$$\text{concentration (μg/g)} = \frac{\dfrac{\text{quantity in the injected sample (μg)}}{\text{injection volume (ml)}} \times \text{final volume of sample (ml)}}{\text{sample weight (g)}}$$

SAMPLE PREPARATION FROM REFINED VEGETABLE OILS

SUPPORT PROTOCOL 1

Most crude food samples contain analytical interference compounds such as proteins and sugars; an extraction procedure is necessary to remove these compounds and concentrate tocopherols and tocotrienols from samples before HPLC analysis. Tocopherols and tocotrienols are fat-soluble and are readily dissolved in organic solvents. Hexane is used to extract tocopherols and tocotrienols from sample matrix. In the extraction, heat is usually applied to the sample to weaken the matrix and allow the solvent to fully access all tocopherols and tocotrienols present. These procedures are described in Support Protocols 2 to 4. However, in refined vegetable oils, because many interference compounds are eliminated during the refining processing, there is no substantial matrix to block organic solvents from extracting any of the components. Thus, extracts from refined vegetable oils can be directly injected into the HPLC system after they are diluted with hexane or mobile phase. As vegetable oils generally possess most forms of tocopherols and tocotrienols, the normal-phase HPLC method is preferable to the reversed-phase HPLC method (see Basic Protocol).

Materials

Refined vegetable oil sample to be analyzed
Hexane (HPLC grade)

16 × 125–mm screw-cap test tube
HPLC sample vials

1. Weigh 0.1 g (exact to 0.001 g) vegetable oil into a 16 × 125–mm test tube.
2. Add 5 ml (exact to 0.01 ml) hexane to the test tube.
3. Vortex 30 sec.
4. Cap the test tube and centrifuge 10 min at 1000 × *g*, room temperature.
5. Transfer a portion of the hexane layer to an HPLC sample vial.

Only a part of the hexane layer is transferred because the volume of HPLC vial is typically less than 2 ml.

Vegetable oil is readily dissolved in hexane. However, some refined oils may still contain trace amounts of hexane-insoluble components that are precipitated on the bottom of the test tube after centrifugation.

As sample preparation for refined oils is straightforward, the final volume of hexane should be identical to that added at the beginning of extraction. It can be used to calculate concentration directly. It is not necessary to evaporate and redissolve as in the other procedures.

6. Inject extract into the normal-phase HPLC system (see Basic Protocol).

SUPPORT PROTOCOL 2

SAMPLE PREPARATION FROM CRUDE OIL AND FAT PRODUCTS

Compared to refined vegetable oils, the compositions of crude vegetable oils and oil and fat products are more complicated. These samples contain proteins, carbohydrates, and minerals that interfere with HPLC separation and reduce the lifetime of the HPLC column. These compounds need to be largely eliminated from the extract before HPLC analysis. Saponification and heating are used to weaken sample matrices to allow the solvent to fully access all tocopherols and tocotrienols of the sample. Liquid/liquid extraction is used to remove these polar compounds from the organic solvent layer that contains tocopherols and tocotrienols. The normal-phase HPLC method is usually used for crude vegetable oils and vegetable oil products; reversed-phase HPLC can be used for animal fat products.

Materials

Crude oil or fat sample to be analyzed
Ascorbic acid (ACS grade)
90.2% (v/v) ethanol (reagent alcohol; HPLC grade)
80% (w/v) potassium hydroxide (KOH; ACS grade)
Nitrogen (UHP grade)
Hexane (HPLC grade)
Mobile phase (see Basic Protocol)

16 × 125–mm screw-cap test tube
70°C water bath
HPLC sample vials

1. Weigh 0.1 g (exact to 0.001 g) sample and 0.05 g ascorbic acid into a 16 × 125–mm test tube.
2. Add 5 ml of 90.2% ethanol and 0.5 ml of 80% KOH to the test tube and vortex 30 sec.

   Reagent alcohol (HPLC-grade) contains 90.2% ethanol, 4.9% methanol, and 4.9% isopropanol.

3. Flush the test tube with nitrogen and cap the tube.
4. Incubate in a 70°C water bath for 30 min, vortexing periodically.
5. Place in an ice bath for 5 min.
6. Add 3 ml deionized water and 5 ml hexane and vortex 30 sec.
7. Centrifuge 10 min at 1000 × *g*, room temperature.
8. Transfer the hexane layer to another test tube.

9. Add 5 ml hexane to the residual and aqueous layer and vortex 30 sec to re-extract.

   The residual is the solid phase that consists of aggregated sample particles on the bottom of the test tube. The aqueous layer is the aqueous phase above the sample particles. They are extracted together by hexane a second time.

10. Centrifuge 10 min at 1000 × *g*, room temperature.
11. Transfer hexane layer to the test tube containing the previous hexane layer (see step 8).
12. Evaporate hexane from the tube under nitrogen flow.
13. Add 1 ml (exact to 0.01 ml) mobile phase and vortex 30 sec to redissolve the extract.
14. Transfer extract to an HPLC sample vial.
15. Inject extract into the normal- or reversed-phase HPLC system (see Basic Protocol).

SAMPLE PREPARATION FROM MEATS

SUPPORT PROTOCOL 3

As the levels of tocopherols and tocotrienols in meat samples are usually lower than in oil and fat samples, a larger sample size is needed in the sample preparation. The meat sample is homogenized to weaken the sample matrix. As in Basic Protocol 2, saponification, heating, and liquid/liquid extraction are used to increase the recovery and remove interference compounds. Satisfactory results can be achieved using a reversed-phase HPLC method.

Materials

Meat sample to be analyzed
Ascorbic acid (ACS grade)
90.2% (v/v) ethanol (reagent alcohol; HPLC grade)
80% (w/v) potassium hydroxide (KOH; ACS grade)
Nitrogen (UHP grade)
Hexane (HPLC grade)
Mobile phase (see Basic Protocol)

16 × 125–mm screw-cap test tube
Mechanical tissue homogenizer
70°C water bath
HPLC sample vials

1. Weigh 0.5 g (exact to 0.001 g) meat sample and 0.05 g ascorbic acid into a 16 × 125–mm test tube.
2. Add 5 ml of 90.2% ethanol to the test tube.

   Reagent alcohol (HPLC-grade) contains 90.2% ethanol, 4.9% methanol, and 4.9% isopropanol.

3. Place the tube in an ice bath and homogenize the meat sample with a mechanical tissue homogenizer for 30 sec.
4. Add 0.5 ml of 80% KOH to the test tube and vortex 30 sec.
5. Flush the test tube with nitrogen and cap the tube.
6. Incubate in a 70°C water bath for 30 min, vortexing periodically.
7. Place in an ice bath for 5 min.
8. Add 3 ml deionized water and 5 ml hexane and vortex 30 sec.

9. Centrifuge 10 min at 1000 × *g*, room temperature.
10. Transfer the hexane layer to another test tube.
11. Add 5 ml hexane to the residual and aqueous layer and vortex 30 sec to re-extract.

    The residual is the solid phase that consists of aggregated sample particles on the bottom of the test tube. The aqueous layer is the aqueous phase above the sample particles. They are extracted together by hexane a second time.

12. Centrifuge 10 min at 1000 × *g*, room temperature.
13. Transfer hexane layer to the test tube containing the previous hexane layer (see step 10).
14. Evaporate hexane from the tube under nitrogen flow.
15. Add 1 ml (exact to 0.01 ml) mobile phase and vortex 30 sec to redissolve the extract.
16. Transfer extract to an HPLC sample vial.
17. Inject extract into the reversed-phase HPLC system (see Basic Protocol).

SUPPORT PROTOCOL 4

SAMPLE PREPARATION FROM CEREALS AND NUTS

Cereals and nuts are blended to fine particles before sample preparation. Saponification, heating, and liquid/liquid extraction are employed in the sample preparation to weaken sample matrix and eliminate interference compounds. Most cereals and nuts contain various forms of tocopherol and tocotrienol. The normal-phase HPLC method is recommended for these types of samples.

Materials

Cereal or nut sample to be analyzed
Ascorbic acid (ACS grade)
90.2% (v/v) ethanol (reagent alcohol; HPLC grade)
80% (w/v) potassium hydroxide (KOH; ACS grade)
Nitrogen (UHP grade)
Hexane (HPLC grade)
Mobile phase (see Basic Protocol)

Food blender
16 × 125 mm screw-cap test tube
70°C water bath
HPLC sample vials

1. Blend sample to fine powder in a food blender.
2. Weigh 0.5 g (exact to 0.001 g) powder and 0.05 g ascorbic acid into a 16 × 125–mm test tube.
3. Add 5 ml of 90.2% ethanol to the test tube.

   Reagent alcohol (HPLC-grade) contains 90.2% ethanol, 4.9% methanol, and 4.9% isopropanol.

4. Add 0.5 ml of 80% KOH (w/v) to the test tube and voretx 30 sec.
5. Flush the test tube with nitrogen and cap the tube.
6. Incubate in a 70°C water bath for 30 min, vortexing periodically.
7. Place in an ice bath for 5 min.
8. Add 3 ml deionized water and 5 ml hexane and vortex for 30 sec.
9. Centrifuge 10 min at 1000 × *g*, room temperature.

10. Transfer the hexane layer to another test tube.
11. Add 5 ml hexane to the residual and aqueous layer and vortex 30 sec to re-extract.

    The residual is the solid phase that consists of aggregated sample particles on the bottom of the test tube. The aqueous layer is the aqueous phase above the sample particles. They are extracted together by hexane a second time.

12. Centrifuge 10 min at 1000 × *g*, room temperature.
13. Transfer hexane layer to the test tube containing the previous hexane layer (see step 10).
14. Evaporate hexane from the tube under nitrogen flow.
15. Add 1 ml (exactly to 0.01 ml) mobile phase to the tube and vortex.
16. Transfer extract to an HPLC sample vial.
17. Inject extract into the normal-phase HPLC system (see Basic Protocol).

COMMENTARY

Background Information

There are four common naturally occurring tocopherols, named α-, β-, γ-, and δ-tocopherol, and four corresponding compounds named α-, β-, γ-, and δ-tocotrienol. All of these have the tocol structure of 2-methyl-2 (4′,8′,12′-trimethyltridecyl)-chroman-6-ol (Figure D1.5.1) and are called vitamin E vitamers. The difference between tocopherol and tocotrienol is that the tocotrienols have three double bonds at the 3′, 7′, and 11′ positions of the isoprenoid side chain, while tocopherols have a saturated isoprenoid side chain. The α, β, γ, and δ forms of tocopherol and tocotrienol depend on the number and position of methyl groups at position 5, 7, and 8 of the chromanol ring (Figure D1.5.2). Two potential stereochemical configurations of each methyl group at 2′, 4′, and 8′ on the isoprenoid side chain produce eight isomeric forms. The term DL-α-tocopherol or all-rac-α-tocopherol refers to a mixture that contains identical amounts of the eight isomers.

Tocopherols and tocotrienols are pale yellow and viscous oils at high concentration. They are readily soluble in nonpolar organic solvents, e.g., hexane and octane. These solvents are usually used to extract tocopherols and tocotrienols from sample matrices. UV spectra of tocopherols and tocotrienols demonstrate that their maximum absorptions are between 280 and 300 nm (Lang et al., 1992). Also, tocopherols and tocotrienols possess intense fluorescence emission at 320 to 330 nm after excitation at 290 nm, due to the chromanol ring (Lang et al., 1992). The unique fluorescence absorption provides a sensitive and specific detection method for analysis of tocopherols and tocotrienols.

In normal-phase HPLC on a silica column, separation is based on the number and position of methyl substituents on the chromanol ring. In reversed-phase HPLC on a C18 column, separation is based on the structure of the side chain and the number of methyl substituents. It is therefore difficult to completely separate β- and γ-tocopherol and β- and γ-tocotrienols by reversed-phase HPLC, because both have the same side-chain structure and number of methyl substituents on the chromanol ring. Only six peaks are usually found in the reversed-phase HPLC method. Thus, reversed-phase HPLC is recommended for samples from animal tissues, which contain little or no β and γ vitamers.

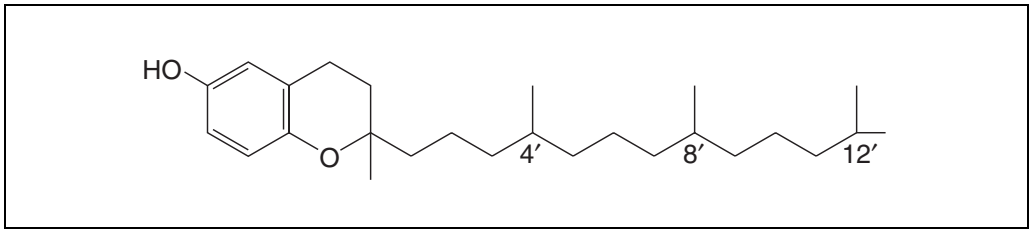

Figure D1.5.1 Structure of tocol: 2-methyl-2-(4′,8′,12′-trimethyltridecyl)chroman-6-ol.

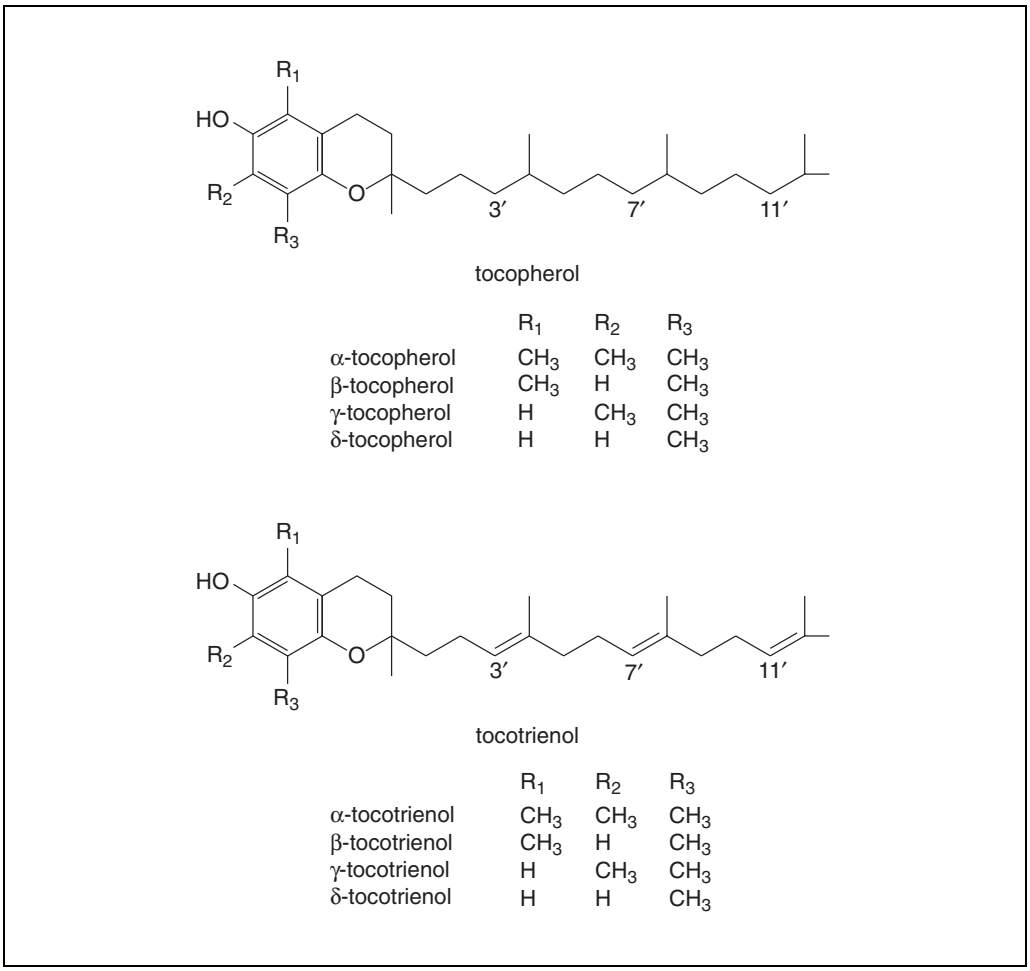

Figure D1.5.2 Structure of tocopherols and tocotrienols.

Critical Parameters and Troubleshooting

HPLC separation

Column. Although the advantage of the normal-phase column is the high resolution in the separation of all tocopherols and tocotrienols, the silica packing material in a normal-phase column is very reactive to strong polar chemicals. Any high-polarity compounds in the sample extract and mobile phase will diminish the column performance and shorten the column lifetime. It is very important to ensure that the sample extract is free of water and metal ions, and it is also advisable to regenerate the column routinely.

Mobile phase. Hexane is the major component of mobile phase in the normal-phase HPLC method. The percentage of hexane is up to 99% for silica normal-phase columns. Ethyl acetate, acetic acid, methanol, and isopropanol are used as modifier components (Shin and Godber, 1994). The flow rate of mobile phase is usually controlled at 1 to 1.5 ml/min to completely separate all eight tocopherols and tocotrienols. It could be increased to shorten the running time after the elution of γ-tocotrienol because the resolution of δ-tocopherol and δ-tocotrienol is more easily achieved compared to the other tocols. In the reversed-phase HPLC method, methanol and acetonitrile are generally the major components in the mobile phase. They can be used individually or together (Tan and Brzuskiewicz, 1989). Common modifier agents in the reversed-phase mobile phase are water, methylene chloride, and acetic acid.

Detectors. Fluorescence and UV detectors are used in the HPLC analysis. The high sensitivity and specificity of fluorescence detection in tocopherols and tocotrienols make the fluorescence detector the first choice. The fluorescence detector is ten times more sensitive and has less background noise than the UV detector. Electrochemical detectors are also used in the analysis of tocopherols and tocotrienols (Murphy and Kehrer, 1987; Sanchez-Perez et al., 2000). As a high-polarity mobile phase is needed for the electrolytes when using an elec-

trochemical detector, the detector can only be applied to a reversed-phase HPLC method. Use of an evaporative light-scattering detector (ELSD) has also been reported in the analysis of tocopherols and tocotrienols (Chase et al., 1994). However, ELSD methods remain to be developed for use with various types of food samples. As the electrochemical and ELSD methods are still not as common as fluorescence and UV detectors in most laboratories and have no significant advantages in food analysis, fluorescence and UV detectors continue to be the predominant detectors used in routine analysis of tocopherols and tocotrienols.

Standards. Pure standard-grade tocopherols (α-, γ-, δ-tocopherol) are available commercially, but tocotrienol standards are not. Shin and Godber (1994) described a method to prepare tocotrienol standards with purity potentially >99% from wheat bran oils.

Sample preparation

Saponification. Before solvent is added for extraction, saponification (alkaline hydrolysis) is a step used in most extractions of tocopherols and tocotrienols. It should be noted that acetate forms of tocopherols or tocotrienols in a sample are changed to free tocopherols and tocotrienols after saponification. This process breaks down the ester bonds of lipids and sample matrices as well. In most extraction procedures, a 60% to 80% (w/v) aqueous solution of KOH is used to perform the saponification. The volume of KOH required varies according to the amount of lipid contained in the sample. Also, ethanol is needed to stabilize the saponified solution and prevent the precipitation of soap material. Usually, the ratio of KOH, ethanol, and fat (in sample) during saponification is 3 (g): 15 (ml): 1 (g), respectively (Ball, 1988). The ratio may need to be adjusted based on the types of fats in the sample. Although ethanol concentration has no effect on the extraction of α-tocopherol by hexane, a concentration above 30% may cause lower recoveries of other tocopherols (Ueda and Igarashi, 1990). For most food samples, saponification for 30 min at 70°C is sufficient.

Lipid extraction prior to saponification is used in some food samples with high concentrations of proteins and carbohydrates. Some of these components are readily hydrolyzed by alkaline solution during saponification, which may generate various hydrolysis products that could interfere with solvent extraction and chromatography. Infant formula is a good example of a case where lipid extraction is performed before saponification. Tuan et al. (1989) indicated that there were fewer interferences when lipid was extracted before saponification than with the normal procedure of saponification before lipid extraction. For food samples with a large quantity of water or lower concentration of tocopherols and tocotrienols, lipid extraction prior to saponification is necessary not only to ensure efficient saponification but also to reduce sample handling size during the remainder of the extraction procedure.

Antioxidants. To reduce oxidation reactions that could impact tocopherols and tocotrienols during saponification and extraction, an antioxidant, such as ascorbic acid or pyrogallol, is added to the sample solution. Also, it is recommended that the sample vessel be flushed with nitrogen and sealed before saponification.

Separation. After saponification, water and organic solvent are added to the sample solution. The solvent is used to retrieve unsaponifiable components into the organic phase. Solvent and aqueous phases are separated after centrifugation or a settling period. The unsaponifiable components, including tocopherols and tocotrienols in sample solution, are extracted into the solvent layer. Most water-soluble interfering substances, including proteins, sugars, and fibers, remain in the aqueous layer. An ice bath could be applied to take advantage of the reduced solubility of interfering substances in the solvent layer at lower temperature (Shin and Godber, 1994).

Hexane is the most common solvent for the extraction. However, the efficiencies of hexane for each tocopherol and tocotrienol are different (Ueda and Igarashi, 1987). α-Tocopherol is completely extracted using hexane alone, but β-, γ-, and δ-tocopherols are not. Hexane mixed with 10% ethyl acetate improves recoveries of β-, γ-, δ-tocopherol (Ueda and Igarashi, 1987). However, ethyl acetate does increase the potential for extraction of some polar interfering substances remaining in the solvent layer. Repeated hexane extraction improves the recoveries of all tocopherols and averts this problem.

Centrifugation is used to separate solvent layer from the extraction system. After centrifugation, there is an observable interface between the organic and aqueous layers due to different light reflection properties of the two phases. The organic layer is transferred into a clean test tube. For a large volume of extract, a separatory funnel is used to remove the aqueous layer. This extraction system involves a settling period in the funnel in order to separate the organic and aqueous layers, which replaces the usual centrifugation. The solvent extraction could be

repeated two or three times to increase the recoveries of all tocopherols and tocotrienols. All of the organic layers are combined together.

Evaporation and redissolving. The solvent of the combined upper layer is evaporated under nitrogen flow or low-temperature vacuum distillation. An oily material appears after it is dried. A precisely measured aliquot of mobile phase is normally used to redissolve the extract. These procedures are intended to not only increase the concentration of tocopherols and tocotrienols to the measurable level of the detector, but also to avoid uncertain volume change of organic layer during extraction, which results in inaccurate results. The redissolved sample is transferred to a vial for HPLC analysis.

Recovery of extraction. The recovery of extraction can be monitored using internal standard and "spiking" methods. For meat products, δ-tocopherol is used as an internal standard because it is usually not detectable in these samples. Ueda and Igarashi (1987) also introduced 2,2,5,7,8-pentamethyl-6-chromanol as an internal standard for the recovery examination. As uneven recoveries occurred in the different forms of tocopherols and tocotrienols during extraction, "spiking," which is adding the standards into the sample prior to saponification and extraction, is a reliable method to obtain the recoveries of the tocopherols and tocotrienols.

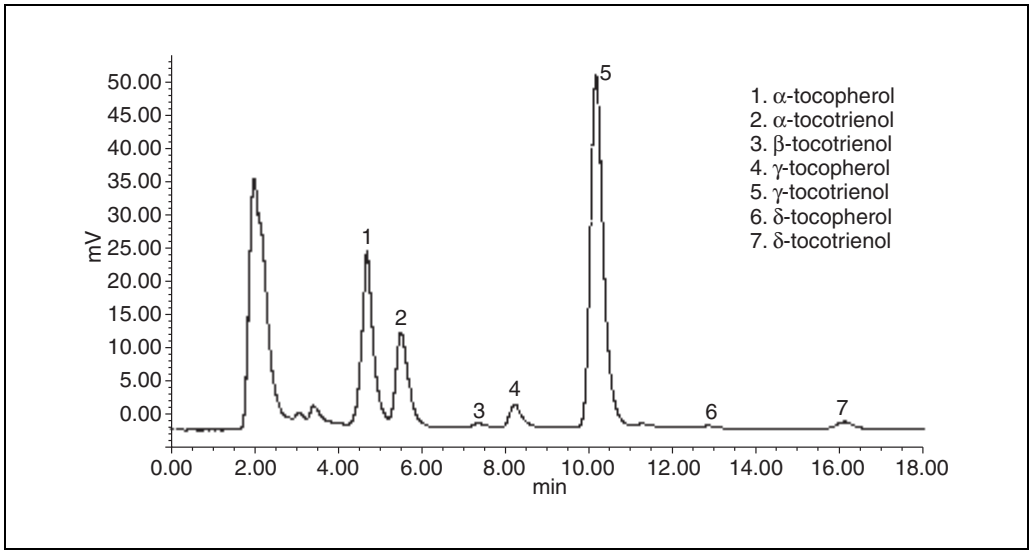

Figure D1.5.3 Chromatogram of tocopherols and tocotrienols of rice bran oil in normal-phase HPLC (see Basic Protocol). β-Tocopherol was below the level of detection.

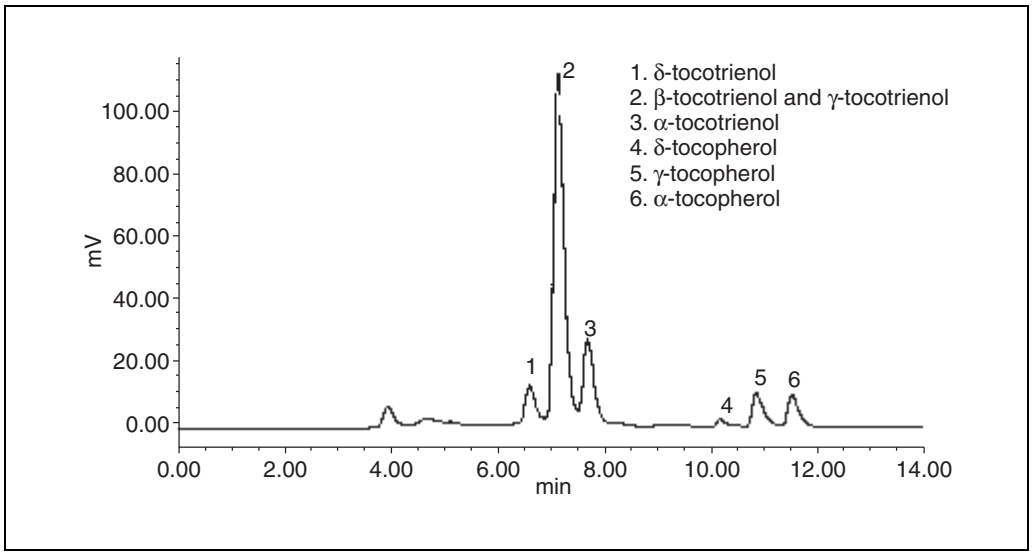

Figure D1.5.4 Chromatogram of tocopherols and tocotrienols of rice bran oil in reversed-phase HPLC (see Basic Protocol). β-Tocopherol was below the level of detection.

Table D1.5.3 Analysis of Tocopherols and Tocotrienols of Foods Using HPLC Methods

Food source	Sample preparation	Column	Detection
Oils and fats	Direct dilution	Tracer Extrasil ODS-2	UV
	Saponification	Tracer Extrasil ODS-2	UV
	Automation	Brownlee Labs RP-18	EC
	Directly dilution	Pentafluorophyenyl column	UV
	Directly dilution	Ultrasphere Silica	ELSD and FL
	Saponification	Supelcosil LC-Si	FL
Vegetables and fruits	Saponification	Rainin Dynamax	UV
	No saponification	Lichosorb	FL
	Saponification	Phenomenex Prodigy ODS-2	UV
Meats	No saponification	Rainin Microsorb Silica	FL
	No saponification	Spherisorb Silica	FL
	Saponification	Waters Silica column	FL
Infant formulas	Solid-phase dispersion	Supelcosil LC-Si	FL
	Saponification	Hypersil silica	UV
Diet	Saponification	Supelcosil LC-18	UV

Anticipated Results

Order of elution and sample chromatograms

In normal-phase HPLC on a silica column, the order of elution of tocopherols and tocotrienols is α-tocopherol, α-tocotrienol, β-tocopherol, β-tocotrienol, γ-tocopherol, γ-tocotrienol, δ-tocopherol, and δ-tocotrienol. Figure D1.5.3 is an example of a normal-phase chromatograph of tocopherols and tocotrienols in rice bran oil.

In reversed-phase HPLC on a C18 column, only six peaks are usually found, and their order of elution is δ-tocotrienol, β- and γ-tocotrienols, α-tocotrienol, δ-tocopherol, β- and γ-tocopherol, and α-tocopherol. Figure D1.5.4 is an example of a reversed-phase chromatograph of tocopherols and tocotrienols in rice bran oil. The tocols with unsaturated side chain have shorter retention time than those with saturated side chain. The methyl substituents on the chromanol ring also affect the retention times of tocopherols and tocotrienols. However, the effect is reversed, compared with the normal-phase HPLC method.

Some of the latest examples of determination of tocopherols and tocotrienols in foods using HPLC are listed in Table D1.5.3.

Calculation of biological activity

α-Tocopherol Equivalent (α-TE) is used to express vitamin E activity in food. The National Research Council defined 1 mg of α-tocopherol as 1 unit of α-TE (mg × 1). The activities as α-TE of other vitamers were: β-tocopherol, mg × 0.5; γ-tocopherol, mg × 0.1; δ-tocopherol, mg × 0.03; α-tocotrienol, mg × 0.3; and β-tocotrienol, mg × 0.05. The activities of γ- and δ-tocotrienol were undetectable. The Recommended Dietary Allowances (RDAs) are only based on intake of the 2R-stereoisomeric forms of α-tocopherol (RRR-, RSR-, RRS-, and RSS-tocopherol) from food, fortified food, and vitamin supplements (Food and Nutrition Board, 2000). The 2S-stereoisomeric forms of α-tocopherol and the other tocopherols (β-, γ-, and δ-tocopherol) and tocotrienols are not used to estimate the RDAs.

Time Considerations

Thirty minutes is sufficient for sample preparation beginning with refined vegetable oils. However, the other sample preparations take up to 120 min because of the incubation, re-extraction, and evaporation steps. Usually, a batch of samples is prepared at the same time to reduce the preparation time per sample. The running time for HPLC analysis is approximately 18 min for normal phase or 14 min for reversed phase in order to quantify all tocols. The running time for normal phase could be cut to 12 min for samples without δ-tocopherol and tocotrienol.

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Quantitation of Lipid Classes by Thin-Layer Chromatography with Flame Ionization Detection

The term lipid is a broad definition for a group of compounds which are extracted into organic solvents such as chloroform or hexane from foods and biological samples. Similar to proteins and carbohydrates, lipids represent one of the major classes of compounds in foods, providing nutritional value and contributing to the sensory, chemical, and physical properties of food products. Lipids are composed of a large number of different classes of compounds, and are analytically classified into neutral (e.g., triglycerides, free fatty acids, diglycerides, monoglycerides, sterols, wax ester, sterol esters, hydrocarbons) and polar lipids (mainly phospholipids). This unit describes the quantitation of different lipid classes. The quantitation of the compositional profile of lipids within each class (except for phospholipids) is a different subject and therefore is not included.

Thin-layer chromatography (TLC) on silica gel is well known for its separation power for lipids and related compounds. The flame ionization detector (FID) is a universal analytical instrument that offers high sensitivity and linearity for carbon-containing organic compounds. The combination of TLC and FID led to the wide use of the Iatroscan TLC-FID for the analysis of lipid classes. The adoption of the Iatroscan TLC-FID in both academia and industry has generated sufficient data to indicate that TLC-FID is currently one of the most efficient tools for the quantitation of lipids classes (Ackman et al., 1990; Hammond, 1993).

Several procedures are used for the quantitation of lipid classes using the Iatroscan TLC-FID system (Iatron Laboratories), but the basic process involves separation of the sample on a thin layer of silica or alumina gel coated on a Chromarod, which is then scanned by the FID. Lipids are separated based on differences in their molecular functional groups, polarity, degree of saturation, and chain length. Selection of the appropriate quantitation procedure depends on the nature of the sample and on the lipid classes that need to be quantified. Neutral lipids are quantified by single development on Chromarods with only a single scan using FID (see Basic Protocol). The obtained results include the amount of individual lipid classes and the total amount of phospholipids. To analyze both neutral and polar lipids (phospholipids), multiple development steps in different solvent systems are carried out, followed by multiple scans of the Chromarods using FID (see Alternate Protocol). In cases where resolution is not optimal due to structural differences among molecules within a given lipid class, a method for hydrogenation of lipids is provided (see Support Protocol).

CAUTION: Due to the hazardous nature of chloroform, procedures using chloroform must be conducted in a chemical fume hood. See *APPENDIX 2B* for more information.

CAUTION: Hydrogen/air mixtures within the flammable range (4% to 74%) can explode with a small amount of ignition energy. Special handling should be taken to prevent mixing of hydrogen with room air. Care should also be taken to eliminate sources of ignition such as open flames, extremely hot objects, or sparks from electrical equipment or even static electricity.

NOTE: All reagents must be at least analytical grade or higher purity.

Contributed by Shengying Zhou
Current Protocols in Food Analytical Chemistry (2003) D1.6.1-D1.6.14

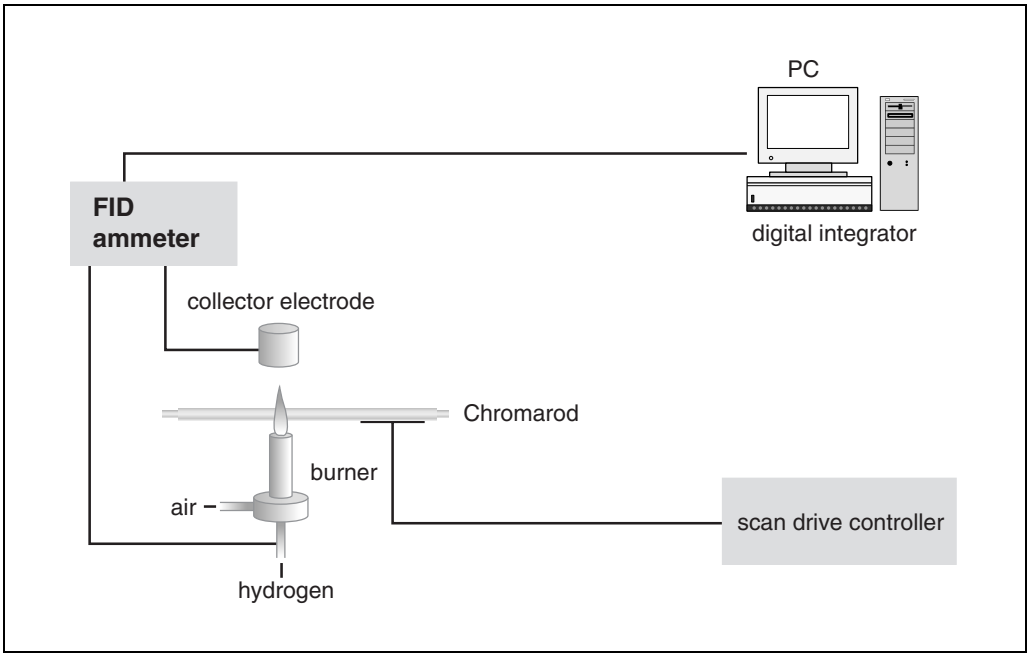

Figure D1.6.1 Diagram of the Iatroscan TLC-FID system. Reprinted with kind permission of SES-Analyses Systems, Germany.

BASIC PROTOCOL

DETERMINATION OF NEUTRAL LIPIDS BY TLC-FID

Figure D1.6.1 outlines the principle of the Iatroscan TLC-FID system. A sample of total lipids (microgram range) is dissolved in organic solvents and spotted onto Chromarods coated with silica or alumina gel. The Chromarod is specially made so that the silica or alumina gel is permanently coated onto a thin quartz rod which can be used repeatedly for numerous analyses. The Chromarods (up to ten) are usually mounted in a metal rack, which is then placed into a solvent system in a closed tank and developed for a fixed period of time. The rack containing the Chromarods is then removed from the tank and dried in a heating oven. The rack is then placed onto the motion frame of the scanning unit, which is just above the hydrogen flame. Once set in motion, the frame of the scanning unit moves automatically in a way that allows the Chromarods to be scanned one by one from top to bottom. As the rods are passed through the hydrogen flame, carbon ions are produced and collected by a metal cylinder (the collector electrode), the current is amplified, and the amplified carbon ion signals are recorded. The advantages of using TLC-FID for lipid analyses can be attributed to its high sensitivity, its resolving power, and speed.

In the protocol described here, lipid compounds on the Chromarods are developed in a single solvent system and the Chromarod is fully scanned from top to bottom over the hydrogen flame. For most lipid samples, the major classes quantified using this single-development, single-scan protocol include triglycerides, free fatty acids, free sterols, sterol esters, diglycerides, and polar lipids (mainly phospholipids). A method for quantification of individual phospholipids is given elsewhere (see Alternate Protocol), and involves the use of a multidevelopment, multiscan procedure.

Materials

97:3:1 (v/v/v) hexane/diethyl ether/formic acid, fresh
Lipid sample
Methylene chloride

Calibration standards (Sigma), for example:
Tripalmitin (triglyceride)
Palmitic acid (free fatty acid)
Nonadecane (hydrocarbon)
Hexadecyl palmitate (wax ester and sterol ester)
Cholesterol (free sterol)
Dihexadecanoyl lecithin (polar lipid)
Hydrogen, high purity

Iatroscan TLC-FID analyzer (e.g., model MKIII; Iatron Laboratories) with Chromarod developing tank
Filter paper, analytical grade
10-ml volumetric flask
Chromarod SIII and Chromarod rack (Iatron Laboratories)
Spotting device: 1-µl disposable micropipet (e.g., Drummond Microcap), 1-µl microsyringe, or (semi)automatic sample spotter
Constant humidity chamber containing a cup of saturated NaCl solution
100°C oven

Equilibrate TLC tank

1. Line a TLC developing tank with clean filter paper. Pour 97:3:1 (v/v/v) hexane/diethyl ether/formic acid (the solvent system) into the tank, wetting the entire paper. Incubate at room temperature until the tank is saturated with the solvent system (at least 1 hr).

   Although a Chromarod developing tank may be provided with the analyzer, any regular rectangular glass TLC tank can be used for development as long as the Chromarod rack fits (e.g., 20 × 20–cm).

   See Critical Parameters and Troubleshooting for a discussion of solvent systems.

Prepare samples and standards

2. Prepare a lipid sample stock solution (~20 mg/ml) by accurately weighing ~200 mg total lipid into a 10-ml volumetric flask. Dilute to the mark with methylene chloride and mix well.

   If a sufficient amount of total lipids is not available, scale down proportionally. Store sample solution up to 48 hr in the refrigerator (~4°C) if it is not analyzed immediately.

3. Prepare calibration standard solutions by dissolving each standard in methylene chloride at 15 µg/µl and then performing serial dilutions at 1, 5, and 10 µg/µl.

   Calibration standards are usually mixed together in one solution if they can be separated from each other by Chromarods. If not, individual solutions of the unresolved standards should be prepared separately. Not all of the standards listed in the materials section are necessary for calibration. Selection of calibration standards depends on the lipid-class profile of each particular sample. Dihexadecanoyl lecithin is used to represent the whole group of phospholipids. See Critical Parameters and Troubleshooting for a discussion on calibration standard preparation and FID linearity.

   The purity of the standards should be checked before use by standard TLC or by analyzing on an Iatroscan TLC-FID system using the same solvent system as the sample.

Perform TLC-FID

4. To ensure they are clean, scan a rack containing ten Chromarod SIIIs on an Iatroscan TLC-FID analyzer using a scanning speed of 4 mm/sec, air flow of 2000 ml/min, and hydrogen flow of 160 ml/min. Scan once more if peaks can be seen in the first scan.

   Prescanning will remove trace amounts of organic compounds deposited during storage and handling. Following repeated use (~30 runs), the Chromarods should be cleaned by soaking overnight in 35:65 (v/v) concentrated nitric acid/water and then washing succes-

sively with tap water, distilled water, and acetone. The clean Chromarods are then dried 30 min in a 110°C oven. Chromarods should be discarded when they show poor performance in separating lipid classes or poor reproducibility.

5. Carefully apply 1 µl of a sample solution onto the starting point (origin) of five Chromarods using an appropriate spotting device. Spot another sample or one concentration of the standard solution onto another five Chromarods.

   The sample solution should be spotted in a way that minimizes the size of the spot, usually in several aliquots (see Critical Parameters and Troubleshooting).

   For each set of Chromarods (normally ten per rack), five are considered as one unit and are spotted with one sample or standard solution to provide five replicates. The average peak area derived from each unit is considered as one data point. One rack (two units) can be processed and analyzed at a time.

6. Place the rack containing the ten spotted Chromarods into a constant humidity chamber containing a cup of saturated NaCl solution and incubate 10 min.

   See Critical Parameters and Troubleshooting for more information concerning Chromarod conditioning.

7. Immediately transfer the Chromarod rack into the equilibrated developing tank (step 1) and incubate 45 min at room temperature.

   The compositional profile of lipid classes varies substantially depending on the sample and its origin. The separation efficiency of lipid classes on Chromarods is affected not only by the solvent system, the operating procedures, the type of Chromarod, and its quality, but also by the lipid class profile of the lipid sample. The above solvent system works well in lipid samples that are low in triglyceride content. For lipid samples high in triglyceride content, the free fatty acid peak and triglyceride peak might not be completely separated since the free fatty acid peak is immediately followed by triglycerides, which can be easily overloaded. Table D1.6.1 provides a list of alternate solvent systems which can be used for lipid separation.

8. Take out the Chromarod rack and dry 3 min in a 100°C oven.

9. Immediately scan the Chromarods on an Iatroscan TLC-FID analyzer using the same settings as in step 4. Record peak areas.

   Figure D1.6.2 shows an Iatroscan TLC-FID chromatogram of lipids from the gastric contents of a hooded seal pup using a solvent system of 91:6:3:1 (v/v/v/v) hexane/ethyl acetate/diethyl ether/formic acid (Ackman and Heras, 1997). Individual lipid classes are clearly separated from one another. Figure D1.6.3 shows an Iatroscan TLC-FID chromatogram of lipid standards using the same solvent system. Figure D1.6.4 shows four Iatroscan TLC-FID chromatograms of hydrogenated cod muscle lipids using four different solvent systems.

10. Repeat steps 4 to 9 as necessary until all samples and standards have been analyzed.

    To reduce rod-to-rod variability, it is best to use the same set of ten Chromarods to analyze all samples and standards (see Critical Parameters and Troubleshooting).

Table D1.6.1 Solvent Systems for Lipid Class Separation on an Iatroscan TLC-FID

Solvent system (v/v)	Development time	Reference
91:6:3:1 hexane/ethyl acetate/diethyl ether/formic acid	—[a]	Ackman and Heras (1997)
80:14:1:0.2 hexane/chloroform/isopropanol/formic acid	55 min	Zhou and Ackman (1996)
60:17:0.5 hexane/diethyl ether/acetic acid	—[a]	Ackman et al. (1990)
98:2:1 hexane/diethyl ether/formic acid	85 min	Ratnayake and Ackman (1985)
85:15:0.04 hexane/diethyl ether/formic acid	40 min	Ohshima et al. (1987)

[a]Development times were not specified by the authors for these systems.

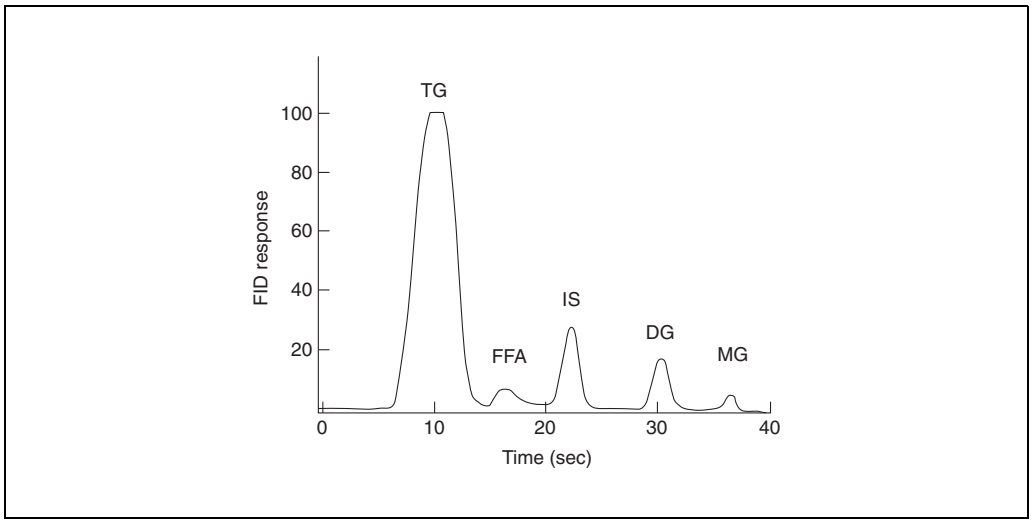

Figure D1.6.2 TLC-FID separation of lipids recovered from the gastric contents of a hooded seal pup. The mobile phase was 91:6:3:1 (v/v/v/v) hexane/ethyl acetate/diethyl ether/formic acid. Time refers to scanning time of the Chromarod. Abbreviations: DG, 1,2-diglyceride; FFA, free fatty acid; MG, monoglyceride; IS, internal standard; TG, triglyceride. Reproduced from Ackman and Heras (1997) with permission from AOCS Press.

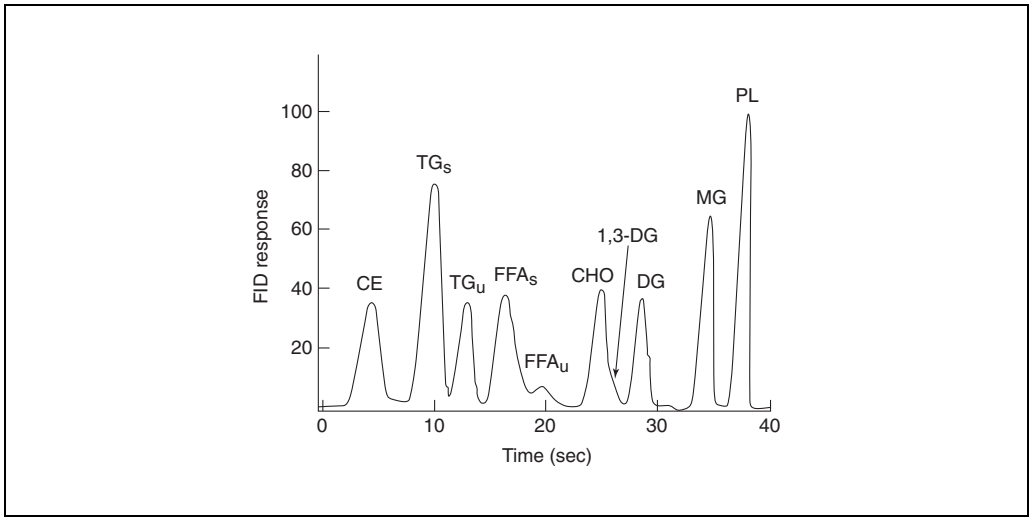

Figure D1.6.3 TLC-FID separation of a range of standard lipids. The mobile phase was 91:6:3:1 (v/v/v/v) hexane/ethyl acetate/diethyl ether/formic acid. Time refers to scanning time of the Chromarod. Abbreviations: CE, cholesterol ester; CHO, cholesterol; DG, 1,2-diglyceride; 1,3-DG, 1,3-diglyceride; FFA_u, highly unsaturated free fatty acid; FFA_s, less unsaturated free fatty acid; MG, 1-monoglyceride; PL, phosphatidylcholine; TG_u, highly unsaturated triglyceride; TG_s, saturated triglyceride. Tripalmitin and palmitic acid were used to complement trilinolenin and linolenic acid. Reproduced from Ackman and Heras (1997) with permission from AOCS Press.

Analyze data

11. Calculate the average peak area for each lipid class from each unit of Chromarods (i.e., for each sample and standard solution).

12. Construct a separate calibration curve for each lipid class by plotting the average peak area of each lipid standard (y) versus concentration in micrograms per microliter (x).

13. Conduct both linear and quadratic regression analyses for each calibration curve. Choose the method which provides the higher R^2 value for quantification of each lipid class in the sample.

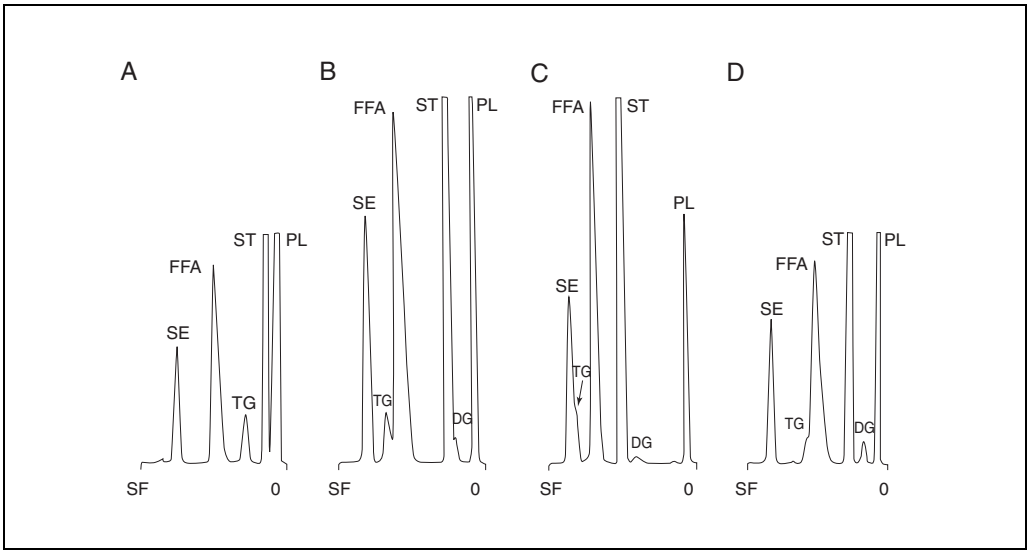

Figure D1.6.4 Iatroscan TLC-FID chromatograms of a hydrogenated lipid fraction isolated from cod flesh (consisting basically of neutral lipids) and separated using several different developing solvent systems: (**A**) 97:3:1 (v/v/v) hexane/diethyl ether/formic acid for 40 min; (**B**) 85:15:0.04 (v/v/v) hexane/diethyl ether/formic acid for 40 min; (**C**) 70:30:1 (v/v/v) benzene/chloroform/formic acid for 40 min; and (**D**) 92:8:0.1 (v/v/v) dichloroethane/chloroform/acetic acid for 30 min. Abbreviations: DG, diglyceride; FFA, free fatty acid; O, origin; PL, phospholipids; SE, steryl ester; SF, solvent front; ST, free sterol; TG, triglyceride. Reproduced from from Ohshima et al. (1987) with permission from AOCS Press.

See Critical Parameters and Troubleshooting for more information concerning calibration standard curves.

14a. *For linear regression:* Calculate the amount of each lipid class from the average peak area (five rods) of each sample from the linear regression equation ($y = ax + b$) and the following equation:

$$\text{lipid class (mg/100 mg sample)} = \frac{(y-b)\times v}{a\times w}\times 100$$

where y is the average peak area of each lipid class (step 11), a is the slope of the regression line for each lipid class, b is the intercept of the regression line for each lipid class, v is the volume of total lipid solution in milliliters (step 2), and w is the weight of the lipid-containing sample in milligrams (step 2).

14b. *For quadratic regression:* Calculate the amount of each lipid class from the average peak area (five rods) of each sample from the quadratic regression equation ($y = ax^2 + bx + c$) and the following equation:

$$\text{lipid class (mg/100 mg total lipids)} = \frac{-b+\sqrt{b^2-4a(c-y)}\times v}{2a\times w}\times 100$$

where a, b, and c are the constants of quadratic regression equation.

DETERMINATION OF BOTH NEUTRAL AND POLAR LIPIDS BY TLC-FID

ALTERNATE PROTOCOL

Both neutral and polar lipids are separated through three development steps in a sequence of three different solvent systems with increasing polarity. Lipids are then revealed by three-step scanning of the separated lipid classes by FID. In a multidevelopment and multiscan system such as this, the scanning distance of each Chromarod is reactivated by the hydrogen flame and compounds in the unscanned area of the Chromarods can be redeveloped in the next solvent system, dried in an oven, and rescanned over the hydrogen flame. The lipid classes quantified in this three-step development and scanning processes are neutral lipids, acetone-mobile lipids, and phospholipids, respectively. This protocol is applicable for lipid samples containing a high percentage of both neutral and polar lipids. If the amount of polar lipids is low (<10%), the accuracy of phospholipid quantitation is likely to be low due to the limitation on FID sensitivity and linearity.

Additional Materials *(also see Basic Protocol)*

80:14:1:0.2 (v/v/v/v) hexane/chloroform/isopropanol/formic acid, fresh
Acetone
70:30:3 (v/v/v) chloroform/methanol/water, fresh
Dihexadecanoyl cephalin (Sigma)

Equilibrate TLC tanks

1. Prepare three developing tanks (see Basic Protocol, step 1) using the following solvent systems:

   80:14:1:0.2 (v/v/v/v) hexane/chloroform/isopropanol/formic acid
   Acetone
   70:30:3 (v/v/v) chloroform/methanol/water.

Prepare samples and standards

2. Prepare lipid sample solution and calibration standard solutions as described (see Basic Protocol, steps 2 and 3) but include dihexadecanoyl cephalin in the standard solution.

   Dihexadecanoyl lecithin is used to represent all individual phospholipids except phosphatidyl ethanolamine, which is calibrated by dihexadecanoyl cephalin.

Perform TLC-FID

3. Scan a set of ten Chromarods, spot with the first set of samples and/or standards, and condition as described (see Basic Protocol, steps 4 to 6).

4. Immediately transfer the Chromarod rack to the first developing tank (containing hexane/chloroform/isopropanol/formic acid) and develop 55 min at room temperature.

5. Take out the Chromarod rack and dry 1.5 min in a 100°C oven.

   In the first and the second development steps, the drying time is reduced in order to avoid the oxidation of polar lipids, which are separated in the third development step.

6. Partially scan the Chromarods (see Basic Protocol, step 9) from the top to a point just below the diglyceride peak to reveal neutral lipids.

   The point on the Chromarod where the partial scanning is stopped should be predetermined in a trial run using a Chromarod or unit of Chromarods containing the same sample. The instrument can be set to automatically scan to the same point on each rod. This setting is based on rod length and should be readjusted each time fresh developing solvents are used.

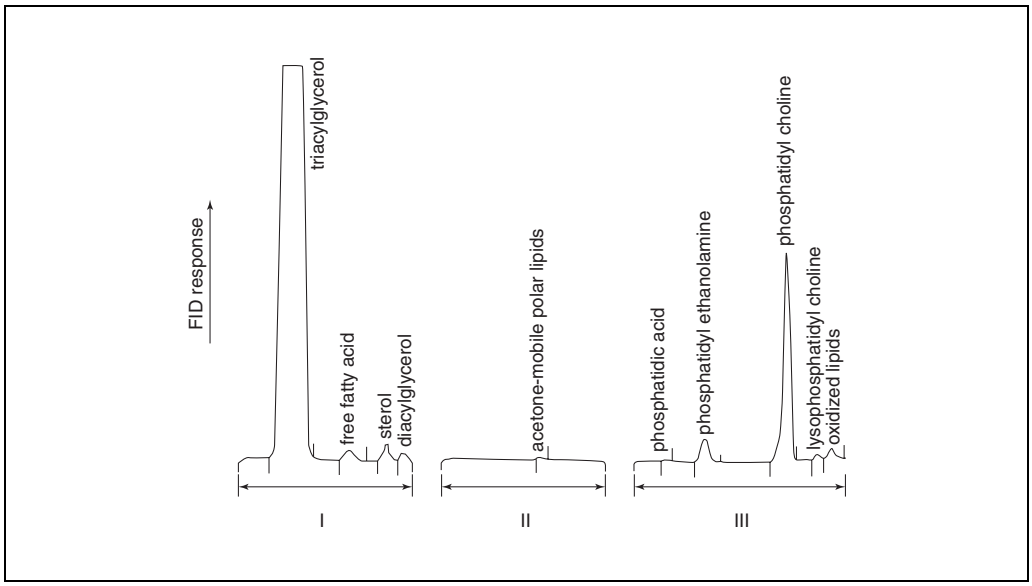

Figure D1.6.5 Sequential Iatroscan TLC-FID profiles of the lipid classes extracted from the dorsal white muscle of Atlantic salmon. I, II, and III represent partial chromatograms from the three-stage development of total lipids on a Chromarod SIII. The solvent systems were: (I) 80:14:1:0.2 (v/v/v/v) hexane/chloroform/isopropanol/formic acid for 55 min; (II) acetone for 15 min; and (III) 70:30:3 (v/v/v) chloroform/methanol/water for 60 min.

7. Immediately transfer the Chromarod rack to the second developing tank (containing acetone) and develop 15 min at room temperature.
8. Take out the Chromarod rack and dry 1.5 min in a 100°C oven.
9. Partially scan Chromarods from the top to a point just below the acetone-mobile lipid peak to reveal acetone-mobile lipids (including monoglyceride).

   Again, the region of partial scanning should be predetermined.

10. Immediately transfer the Chromarod rack to the third solvent tank (containing chloroform/methanol/water) and develop 60 min at room temperature.
11. Take out the Chromarod rack and dry 3 min in a 100°C oven.
12. Completely scan the Chromarods to reveal phospholipids.
13. Repeat steps 3 through 12 with any remaining samples and standards.

    Figure D1.6.5 shows Iatroscan TLC-FID chromatograms of lipids from dorsal white muscle of Atlantic salmon obtained using this three-solvent, three-step scanning procedure.

Analyze data

14. Prepare calibration curves and calculate lipid class concentrations as described (see Basic Protocol, steps 11 to 14).

QUANTITATION OF LIPID CLASSES AFTER HYDROGENATION

SUPPORT PROTOCOL

Silica-gel Chromarods are very effective in separating compounds that differ in molecular structure. Typically, compounds within a single lipid class are similar in structure, but differ primarily in chain length and in saturation (i.e., in the number of double bonds). In some cases, however, the separation power of Chromarods is strong enough that compounds within a single lipid class are separated, causing partial separation of peaks or peak broadening of that lipid class (e.g., palmitic acid 16:0 versus docosahexenoic acid 22:6; Fig. D1.6.3). Hydrogenation of total lipids can reduce the structural difference between molecules within each lipid class, thus providing significant peak narrowing and improving the separation between lipid classes. The result is increased accuracy and precision for lipid class quantitation.

In the following procedure, lipids are dissolved in solvent and hydrogenated in the presence of hydrogen and a catalyst (platinum oxide). After the hydrogenated lipid solution is processed by filtration and evaporation, it can be analyzed by TLC-FID.

Materials

Lipid sample
Chloroform
Platinum oxide, analytical grade
Hydrogen, chromatographic grade

125-ml flat-bottom flask with standard taper neck
Gas inlet/exit assembly: glass two-hole stopper with glass inlet and exit tubes
Hydrogen flowmeter
Filter paper
Rotary evaporator equipped with an aspirator

1. Weigh ~100 mg lipid sample into a 125-ml flat-bottom flask with standard taper neck containing a Teflon-coated magnetic stir bar. Add 70 ml chloroform and 5 mg analytical-grade platinum oxide (catalyst).

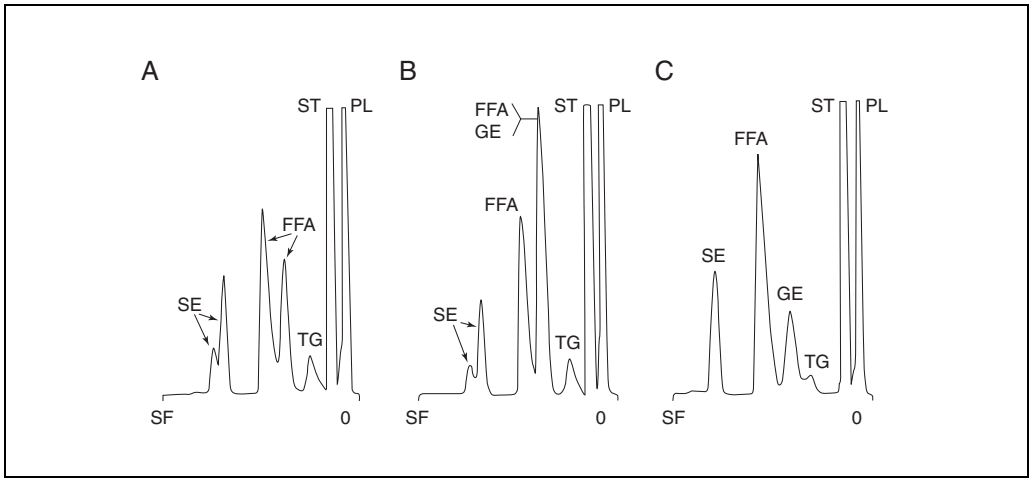

Figure D1.6.6 Iatroscan TLC-FID chromatograms of (**A**) a lipid fraction enriched with neutral lipids isolated from cod flesh and stored in ice; (**B**) neutral lipids spiked with authentic 1-0-palmityl-glyceryl ether dipalmitate (GE), coinciding in position with authentic highly unsaturated acids such as 22:6n-3; (**C**) hydrogenated neutral lipids spiked with GE. The solvent system was 97:3:1 (v/v/v) hexane/diethyl ether/formic acid for 40 min. Abbreviations: O, origin; SF, solvent front; FFA, free fatty acid; PL, phospholipids; SE, steryl ester; ST, free sterol; TG, triglyceride. Reproduced from Ohshima et al. (1987) with permission from AOCS Press.

2. Connect a gas inlet/exit assembly to a chromatographic-grade hydrogen source and adjust the flow of hydrogen to ~2 liters/min using a hydrogen flowmeter. Place the glass stopper with the inlet/exit assembly onto the flask so that the end of the inlet tubing is just above the surface of the solvent.

   CAUTION: *For safety, the hydrogenation process should be carried out in a chemical fume hood, and the exit line should be placed so as to ensure that the waste gas is expelled through the hood's exhaust system.*

3. Begin to gently stir the chloroform in the flask and hydrogenate the sample 50 min at room temperature with continued stirring. Add more chloroform if the level becomes low (<40 ml).

4. Remove catalyst by passing the solution through filter paper (by gravity). Rinse flask and filter paper twice with 10 ml chloroform.

5. Remove chloroform from lipids using a rotary evaporator equipped with an aspirator.

6. Redissolve hydrogenated lipids in 4 ml chloroform.

   The sample is now ready for analysis using the procedures described above (see Basic Protocol and Alternate Protocol). Figure D1.6.6 shows chromatograms of cod muscle lipids before and after hydrogenation. Note the single peaks for steryl esters and free fatty acids in panel C compared to the double peaks in panels A and B.

COMMENTARY

Background Information

Thin-layer chromatography (TLC) on silica gel offers tremendous separation power and speed for analyzing lipid compounds, but the quantitative work can be tedious, costly, and prone to error. Thus, it is not an easy or accurate method for the quantitation of lipid classes. The same problems also exist for high-performance liquid chromatography (HPLC), which still lacks a sensitive and reliable detector to accurately quantitate the lipid compounds eluted from the column. Spectrophotometric detection does not work for lipids since their absorption in the UV/visible region is rather poor. A variety of detectors have been tried for lipid quantitation (e.g., refractive index, evaporative light scattering, flame ionization detectors), but they either lack sensitivity or operate unreliably due to the large quantity of coexisting solvents. A gas/liquid chromatograph (GLC) coupled with a flame ionization detector (FID) is an ideal tool for quantitation of volatile compounds, including the widely accepted analysis of fatty acid profile in lipids. Unfortunately, most of the major lipid compounds have very high boiling points, rendering GLC useless for the quantitation of lipid classes (Hammond, 1993).

The combination of TLC with FID provides an effective solution for the quantitation of lipid classes. This idea was commercialized in Japan in the 1970s with the development of the Iatroscan TLC-FID analyzer and accompanying Chromarods. Since then, both the Chromarods and the instrument have been significantly improved, and their application in various analytical areas has been evaluated (Ackman et al., 1990). The Iatroscan TLC-FID is currently one of the most widely used tools for quantitation of lipid classes.

The Chromarod consists of a straight quartz rod coated with an adsorbing material, usually silica gel, which can be briefly exposed to a hydrogen flame. The sensitivity of the analysis depends primarily upon the proportions of ionizable carbon atoms in the compound being examined. The system is rapid, sensitive, and simple for the analysis of lipid classes.

Good review articles on the evaluation of the Iatroscan TLC-FID include those by Ackman et al. (1990), Indrasena et al. (1991), Shantha (1992), and Hammond (1993). Numerous publications address its application for lipid quantitation of samples including marine lipids (Ohshima et al., 1987; Gunnlaugsdottir and Ackman, 1993; Zhou et al., 1995) and canola gum (Ratnayake and Ackman, 1985).

Critical Parameters and Troubleshooting

Iatroscan TLC-FID instrument and Chromarods

The suggested concentrations of standard and lipid-sample solutions described in both the Basic and Alternate Protocols are based on the characteristics and sensitivity of the Iatroscan TLC-FID Model MK III. Later models of the instrument (e.g., MK IV, MK V, MK VI) are improved in their sensitivity in detecting lipid compounds through FID. As a result, the concentration of standard and lipid-sample solutions should be reduced accordingly when later models are used. The MK VI is the newest model and contains both an FID and an FPD (flame photometric detector). The FPD is specifically designed to selectively detect heteroatoms such as phosphorous and sulfur, which could be useful for quantifying phospholipids. The MK VI is also capable of acquiring data simultaneously by both FID and FPD.

In addition to different types of Chromarods, there are different versions of the same type available on the market. Chromarods coated with alumina and silica gel are both available, but Chromarods coated with alumina are rarely used in lipid analysis due to poor performance (Indrasena et al. 1991). The chromarod SIII (an improved version of the SII) is coated with silica gel and is the most recent and most widely used version of Chromarod.

Calibration standard curves

It is widely accepted that the GLC-FID system is generally high in both linearity and sensitivity when carbon-containing organic compounds are analyzed within a certain loading range of the column. Due to the structural difference between the detectors of the GLC-FID and the TLC-FID in capturing ions, the linearity of the TLC-FID system has never achieved the same standard as the GLC-FIDs and is relatively poor. Later models of the Iatroscan TLC-FID system have improved detector design and claimed to give better linearity and sensitivity. The calibration standard described in this unit is optimal for the Iatroscan model TH-10 MK III. For higher Iatroscan models, the concentration of both standard and sample solutions in this unit could still be applicable, but might not be optimal; therefore, it would be helpful to slightly adjust the range of calibration standards and lipid sample solutions accordingly.

Nonlinear correlation of peak area versus loading of lipid classes may be seen when a wide range of calibration standard concentrations is used (generally by a factor of ten or greater of the highest versus the lowest concentration; Shantha, 1992; Ackman and Heras, 1997). In this case, researchers found that a quadratic regression curve was a better fit than the linear regression line (Parrish and Ackman, 1985; Ackman and Heras, 1997). However, the linear regression line is still a good fit when a narrow range of calibration standard solutions is selected for a particular lipid class. For calculation of lipid-class content, formulas derived both from linear regression and quadratic regression are included in this unit (see Basic Protocol), and the regression curve with the higher R^2 value should be used for the calculation of lipid class content.

Lipid samples from natural sources generally contain various classes of lipid compounds in which concentration of individual lipid class varies substantially. Lipids from fat-rich tissues of biological samples are usually dominated by triglycerides. On the other hand, those from low-fat tissues tend to have even distribution of compounds among lipid classes. The range of calibration standard solutions described in this unit is the same for all lipid classes. To improve the accuracy in lipid-class quantification, it would always be a good approach to adjust the range of individual calibration standards based on the lipid class profile of lipids from particular sources.

Chromarod conditioning

The amount of water in the silica gel on the surface of the Chromarods strongly affects the separation of lipid classes. The moisture content of the silica gel is dependent on the environmental humidity, the history of rod cleaning, the precleaning processes, and the time between prescanning and the beginning of solvent development. It is important to regulate the moisture content of Chromarods before solvent development in order to achieve consistent and reproducible separation of organic compounds, including lipid classes.

To provide constant humidity, Chromarods that have been spotted with a lipid sample are incubated in a constant humidity chamber for a fixed period of time (10 to 15 min) to reach equilibrium before being transferred to a developing tank. A saturated sodium chloride solution is widely used to provide constant humidity, as saturated solutions of inorganic salts give

constant humidity at a constant temperature in a closed tank.

Spotting onto Chromarods

Separation and quantitation of lipid classes on Chromarods depends on a variety of factors, including the actual spotting of the 1-µl sample. The accuracy and precision of lipid class analyses by TLC-FID depends largely on the accuracy and precision of spotting. Drummond Microcap disposable pipets have been widely used in lipid analyses and are preferred to a microsyringe for this purpose. Spreading of the sample around the original spot can be problematic, especially when large volumes need to be spotted. Spreading results in peak broadening, and in some cases, each peak will appear as a doublet. To minimize the spreading of samples on Chromarods, several practical approaches have been devised, including the selection of volatile solvents with high lipid solubility and spotting samples in multiple aliquots (i.e., spotting an aliquot and allowing the solvent to evaporate completely before spotting another aliquot). An inexperienced analyst should practice the spotting procedure until accurate, reproducible, unspread spots can be reliably achieved.

Oxidation of lipids on Chromarods

Microgram levels of lipids are exposed to large quantities of air and to high temperature after they are spotted onto Chromarods. This is especially true when multidevelopment techniques are applied (see Alternate Protocol) and when the degree of unsaturation in the lipid classes is high (e.g., marine lipids). The outcome of these inevitable factors is the oxidation of lipids on Chromarods during analysis, which can alter the lipid class profile. Peaks have been observed at the site of the original spot on Chromarods, which can be in part attributed to the oxidation of lipids (Ratnayake and Ackman, 1985). Every effort should be made to minimize oxidation during analysis. The general precautions include minimizing air contact, and drying Chromarods at lower temperature and for a shorter period of time, as long as the background noise of FID is acceptable.

Variation between rods

One of the problems in applying TLC-FID is the variation in separation and relative responses of lipid classes from rod to rod. This is why several rods on the same Chromarod rack are normally spotted as one unit (i.e., with the same sample) and the average of their peak areas is considered as one data point. The effect of rod-to-rod variation can be minimized by analyzing both the standards and the samples on the same set of rods under identical conditions and by using an internal standard. However, selection of internal standards is, to a large extent, dependent on the type of lipid sample(s) and the analytical objectives (Parrish and Ackman, 1985; Walton et al., 1989; Ackman et al., 1990). Unfortunately, there are no universal internal standards available for the quantitation of lipid classes. Rod-to-rod variation can also be reduced by selecting Chromarods of similar characteristics (e.g., resolving power, response of calibration standard, retention time) from larger batches and by carefully avoiding contacting the Chromarod surface with the micropipet while spotting samples.

Sample load

A sample load of 30 µg total lipids is the upper limit for a Chromarod SIII. Overloading with a higher total lipid amount is likely to affect the resolution between triglycerides and nearby lipid classes, particularly free fatty acids. These two types of lipid classes are chromatographically very close to each other on silica gel Chromarods. Loading Chromarods with higher total lipid amounts usually results in the overloading of triglycerides, which are the major lipid class in most lipid samples.

Peak splitting

Each lipid class is composed of numerous compounds of the same type. The strong separating power of silica gel sometimes splits compounds from the same lipid class into two peaks, or seriously broadens the peak of a specific lipid class (Fig. D1.6.3). Typical examples of this problem include marine lipids, in which fatty acids range from palmitic acid with 16 carbons and no double bonds (16:0) to docosahexenoic acid with 22 carbons and 6 double bonds (22:6; Ackman and Ratnayake, 1989). Peak splitting and/or broadening on Chromarods is a serious problem which could render the accurate quantitation of lipid classes impossible. This problem can sometimes be solved by adjusting or changing the solvent system. If the problem persists, hydrogenation of the total lipid sample (see Support Protocol) is an effective solution (Fig. D1.6.6).

Developing solvent system

Although use of a constant humidity chamber significantly improves the reproducibility of lipid class separation by Chromarods, minor

variations in their separation can occasionally be seen due to environmental humidity changes and other factors such as the freshness of the solvent system, quality of individual solvents, and precision in preparing the developing solvent systems. In some cases, the presence of minor solvent compounds, such as formic acid, is very important in resolving peaks between lipid classes. It is always a good practice to use solvents that are as fresh as possible and to premix solvent systems before pouring them into the developing tank. The solvent system should be replaced after 4 hr of use. Both the filter paper used to line the tank and the tank itself should be free of any trace solvents before pouring in the fresh solvent.

Anticipated Results

Chromarod FID peaks of sterols, diglycerides, monoglycerides, and polar lipids are narrower and sharper than peaks of triglycerides and free fatty acids when analyzed using either method described in this unit (see Basic Protocol and Alternate Protocol). Hydrogenation of total lipids (see Support Protocol) results in much sharper and narrower peaks, which in turn substantially improves the resolution between lipid classes. The accuracy and precision in quantitating lipid classes of vegetable oils and animal fats are expected to be better than those from marine lipids.

One of the disadvantages of using TLC-FID in the quantitation of lipid compounds is the low precision in spotting fixed volumes of sample solution onto the Chromarods, which results in variations in the quantitation of lipid classes. This problem can be minimized if all calibration standards are dissolved in one solution and if calibration is performed using the same set of Chromarods used for the lipid samples.

A peak is always present at the site of the original spot on the Chromarod when using the Alternate Protocol, especially when analyzing oxidized lipids. This peak can be partly attributed to the oxidation of lipids prior to and during the analysis of lipid classes by TLC-FID.

The accurate quantitation of minor lipid classes depends not only on the concentration of total lipids in the sample, but also on the profile of lipid classes in the sample. If one lipid class (e.g., triglycerides) dominates all others, it would be difficult to simultaneously quantify the minor lipid classes due to the overloading problem of the dominating lipid class.

It is not known whether hydrogenation in the presence of catalyst (see Support Protocol) causes side reactions other than the saturation of double bonds. Therefore, this method should only be used if the accurate quantitation of lipid classes cannot be achieved using the Basic Protocol or Alternate Protocol.

Time Considerations

One of the major advantages in using TLC-FID for the analysis of lipid classes is its speed. Fresh developing solvent system should always be prepared at the beginning of the experiment, which takes ~5 min. Spotting of the sample solutions should not be rushed, as it needs to be done very carefully. Once the sample solution has been spotted, the subsequent development and scanning processes should be carried out continuously; no stopping is permitted. The times expected for conducting one analysis by an experienced researcher following the Basic and Alternate Protocols are ~95 min and 200 min, respectively, not including standard calibration. About 190 and 380 min additional time are needed to calibrate four standard solutions.

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This reference fully describes the fundamentals of the Iatroscan TLC-FID system and its application in lipid analyses.

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This is a good reference detailing the comparison of Chromarod SIII (silica gel) and Chromarod A (alumina) in their ability to separate various lipid classes.

Shantha, 1992. See above.

The characteristics, advantages, and disadvantages of the Iatroscan TLC-FID system are fully discussed in this reference. It also contains rich information on the applications of the Iatroscan TLC-FID system in the area of lipids.

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Infrared Spectroscopic Determination of Total *Trans* Fatty Acids

BASIC PROTOCOL

This unit describes the attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopic method (AOCS, 1999a; AOAC International, 2000), a novel method for measuring the total amount of fat with isolated *trans* double bonds. It is applicable to natural fats (ruminant fats) and processed fats and oils (partially hydrogenated fats and oils or refined vegetable oils) consisting of long-chain fatty acid methyl esters or triacylglycerols with *trans* levels ≥5%, as percent of total fat (AOAC International, 2000).

The fatty acid composition of the *trans*-free reference fat plays a critical role. If the selected *trans*-free fat is significantly different from the matrix of the fat investigated, it may have an adverse impact on accuracy, particularly near the official method's lowest *trans* level of quantitation, 5% (AOAC International, 2000) or 1% (AOCS, 1999a). This *trans*-free reference fat must be carefully selected and should represent as much as possible the composition of the unknown *trans* fat or oil being determined.

Materials

Trielaidin (TE) with purity of >99%
Trans-free reference fat (see Critical Parameters)
Test sample

10-mL beakers
Analytical balance (60 g capacity, capable of weighing 0.3 ± 0.0001 g)
Plastic vials with screw caps
Heated attenuated total reflection (ATR) infrared cell (see Critical Parameters)
FTIR spectrometer (see Critical Parameters)
Low-lint tissue paper
Boiling water bath

Prepare calibration standards and warm up instrument

1. Prepare neat (solvent-free) *trans* calibration standards by weighing accurately, on an analytical balance (to the nearest 0.0001 g), 0.3 − *x* g of a *trans*-free reference fat and *x* g of trielaidin (TE) into each of a series of 10-mL beakers, where *x* equals 0.0015, 0.0030, 0.0150, 0.0300, 0.0600, 0.0900, 0.1200, and 0.1500 g. Store calibration standards in plastic screw-cap vials.

   This yields 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, and 50% trans calibration standards, respectively. Neat fat should not be stored in glass vials, as trace metals in the glass will accelerate the oxidation process.

2. Allow the ATR cell to warm up to 65° ± 2°C for 30 min.

Collect data

3. Using a disposable pipet, place 1 to 50 µl of the *trans*-free reference fat on the ATR horizontal surface, making sure that the surface of the ZnSe or diamond crystal is *completely* covered.

4. Collect a 128-scan single-beam FTIR spectrum at 4 cm^{-1} resolution according to the manufacturer's instructions, and save it (Fig. D1.7.1B).

5. Clean the ATR crystal by thoroughly wiping it with low-lint tissue papers as many times as needed. To ensure absence of cross-contamination, apply the next test sample

Contributed by Magdi M. Mossoba and Richard E. McDonald
Current Protocols in Food Analytical Chemistry (2003) D1.7.1-D1.7.7

to be analyzed, and then clean the crystal again with low-lint tissue papers as many times as needed.

6. Melt calibration standards (from step 1) in a water bath if necessary. Using a disposable pipet, place each standard on the ATR horizontal surface making sure that the surface of the ZnSe or diamond crytal is *completely* covered, collect a 128-scan single-beam FTIR spectrum (Fig. D1.7.1B) at 4 cm^{-1} resolution, and save it. Repeat step 5 after each measurement.

7. Melt the *trans* fat test samples in a water bath if necessary. Using a disposable pipet, place each on the ATR horizontal surface making sure that the surface of the ZnSe or diamond crytal is *completely* covered, collect a 128-scan single-beam FTIR spectrum (Fig. D1.7.1B) at 4 cm^{-1} resolution, and save it. Repeat step 5 after each measurement.

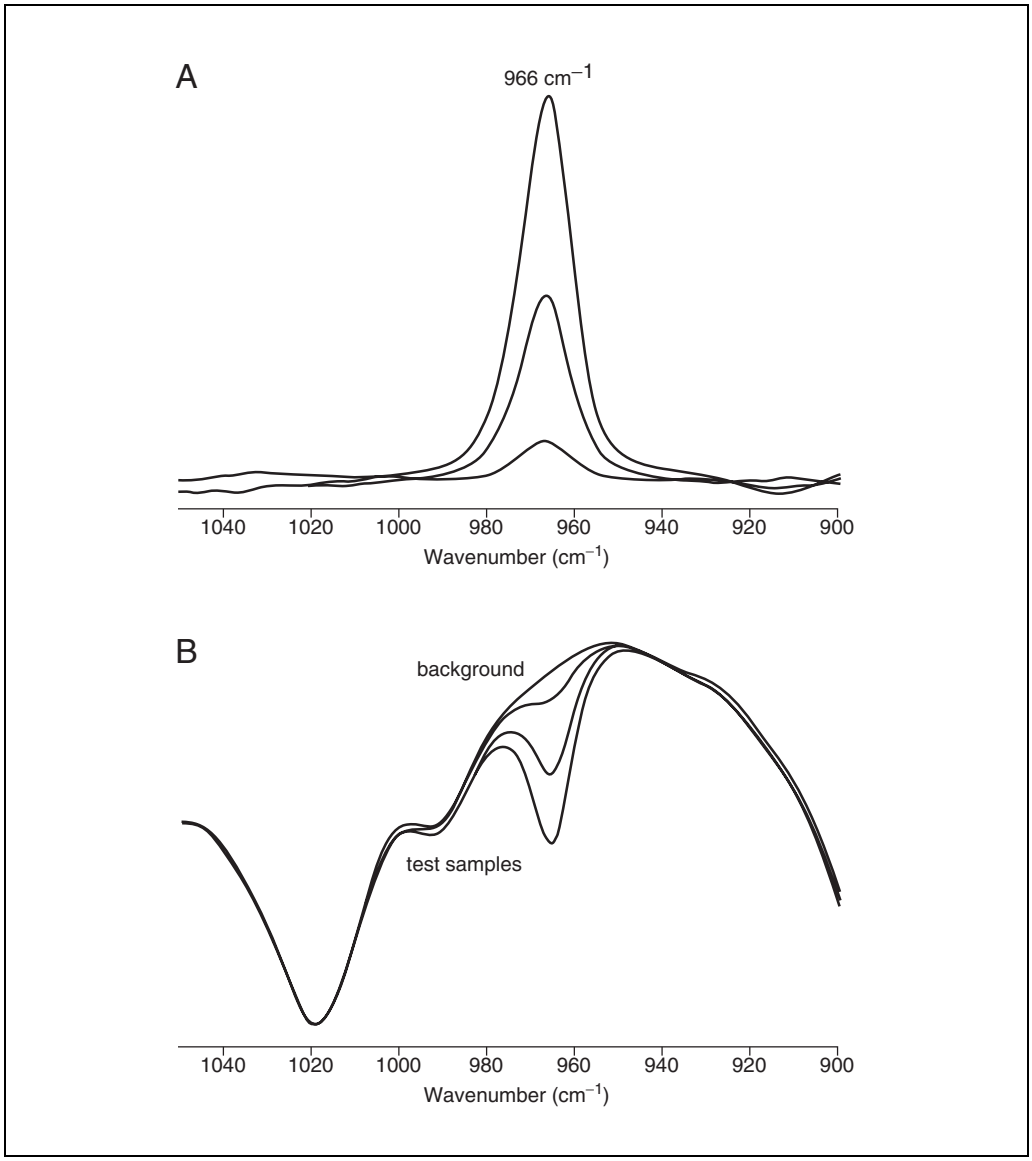

Figure D1.7.1 (**A**) Absorption spectra for neat (without solvent) *trans* fat (test samples). (**B**) Single-beam spectra for neat (without solvent) *trans*-free fat (background) and *trans* fat (test samples). Reprinted from Adam et al. (2000) with permission from the American Oil Chemists' Society.

8. Electronically generate the absorbance spectra (Fig. D1.7.1A) for all the calibration standards and the *trans* fat test samples by "ratioing" each of the saved single-beam spectra against that of the *trans*-free reference fat.
9. For each of the calibration standards and the *trans* fat test samples, display the absorbance spectrum in the expanded wavenumber range of 1050 to 900 cm^{-1}, and electronically integrate the area under the 966 cm^{-1} band between the 990 and 945 cm^{-1} limits.

Calculate results

10. Create a calibration curve by performing a linear regression analysis of the area under the 966 cm^{-1} band versus the amount of TE (as percent of total fat) in the calibration standards.

    Calibration curves should be checked periodically to ensure that they have not shifted.

11. Using the slope and intercept of the linear regression equation generated for the calibration standards, calculate the *trans* level (as percent of total fat) for each test sample by substituting the *trans* band integrated area into the equation:

    % *trans* = (area – intercept)/slope.

    Report results to the nearest 0.1%.

    This equation assumes that the test samples consist of TE.

COMMENTARY

Background Information

Traditional infrared methods for trans fatty acids

Infrared (IR) determinations have traditionally been carried out in the transmission mode (AOAC International, 1997; AOCS, 1999b). The determination of total *trans* triacylglycerols or fatty acid methyl esters (AOCS, 1999a, AOAC International, 2000) is based on the C-H out-of-plane deformation band observed at 966 cm^{-1} (Fig. D1.7.2A) that is uniquely characteristic of isolated double bonds with *trans* configuration. These double bonds are found mostly in *trans*-monoenes (e.g., *trans*9-18:1), and usually at much lower levels in minor hydrogenation products such as methylene-interrupted (e.g., *trans*9,*trans*12-18:2) and non-methylene-interrupted (e.g., *trans*9,*trans*13-18:2) *trans*,*trans*-dienes, mono-*trans*-dienes (e.g., *trans*9,cis12-18:2), and other *trans*-polyenes (e.g., *trans*9,cis12,cis15-18:3). Transmission IR and FTIR methodologies have been extensively used in the fats and oils industry for the determination of fatty acid methyl esters (FAMEs). However, samples consisting of free fatty acids must first be esterified, particularly at low *trans* levels (<15%; Firestone and Sheppard, 1992), because the band near 935 cm^{-1}, due to the O-H out-of-plane deformation in –COOH groups, would interfere with the determination of the *trans* band at 966 cm^{-1}.

When the total isolated *trans* fat levels are relatively low (below 10%), a potentially significant interference may be found in products containing conjugated unsaturation, e.g., 1% in ruminant fat or 5% in tung oil (Firestone and Sheppard, 1992). This is due to the fact that conjugated *trans,trans* (near 990 cm^{-1}) and/or *cis/trans* (near 990 and 950 cm^{-1}) double bonds exhibit absorption bands that are sufficiently close to 966 cm^{-1}. This interference can be eliminated by applying a standard addition procedure (Mossoba et al., 2001a). This procedure also applies to interferences from the food matrix.

Using 966 cm^{-1} absorption to determine total trans fat

Using internal reflection, also known as attenuated total reflection (ATR), an official ATR-FTIR method (AOCS, 1999a; AOAC International, 2000) was recently developed (Mossoba et al., 1996, 2001b; Adam et al., 2000) to rapidly (5 min) measure the 966 cm^{-1} *trans* band as a symmetric feature on a *horizontal* baseline (Fig. D1.7.1A). The experimental aspects of this ATR infrared official method are far less complex than those involving the conventional transmission measurements. This approach entails (1) "ratioing" the *trans* test sam-

ple single-beam spectrum against that of a reference material consisting of a *trans*-free oil, and (2) applying the horizontal ATR sampling technique (Harrick, 1967; Mirabella, 1992; Ismail et al., 1998) to melted fats to avoid the weighing of test portions and their quantitative dilution with the volatile carbon disulfide (CS_2) solvent. This ATR sampling technique allows *trans* reference materials and test samples that consist of any neat (without solvent) melted (at ~65°C) fats or liquid fats to be accurately measured without the time-consuming requirement of having to quantitatively prepare fat solutions.

The extraction of fats that are free of contaminants from different food matrices depends on the nature of the food matrix being investigated. Official method AOAC 996.06 (AOAC, 1997) describes procedures for the extraction of fat and fatty acids from foods by hydrolytic methods. These include acidic hydrolysis for most foods, alkaline hydrolysis for dairy products, and a combination of these procedures for cheese. To minimize oxidative degradation of fatty acids during analysis, this method requires the addition of pyrogallic acid. A detailed discussion of the extraction of fat from foods is beyond the scope of this infrared spectroscopic determination.

In FTIR instrumentation, single-beam spectra (Fig. D1.7.1B and D1.7.2B) are measured separately for both a test sample and an appropriate reference background material, and then "ratioed" to obtain an absorption spectrum

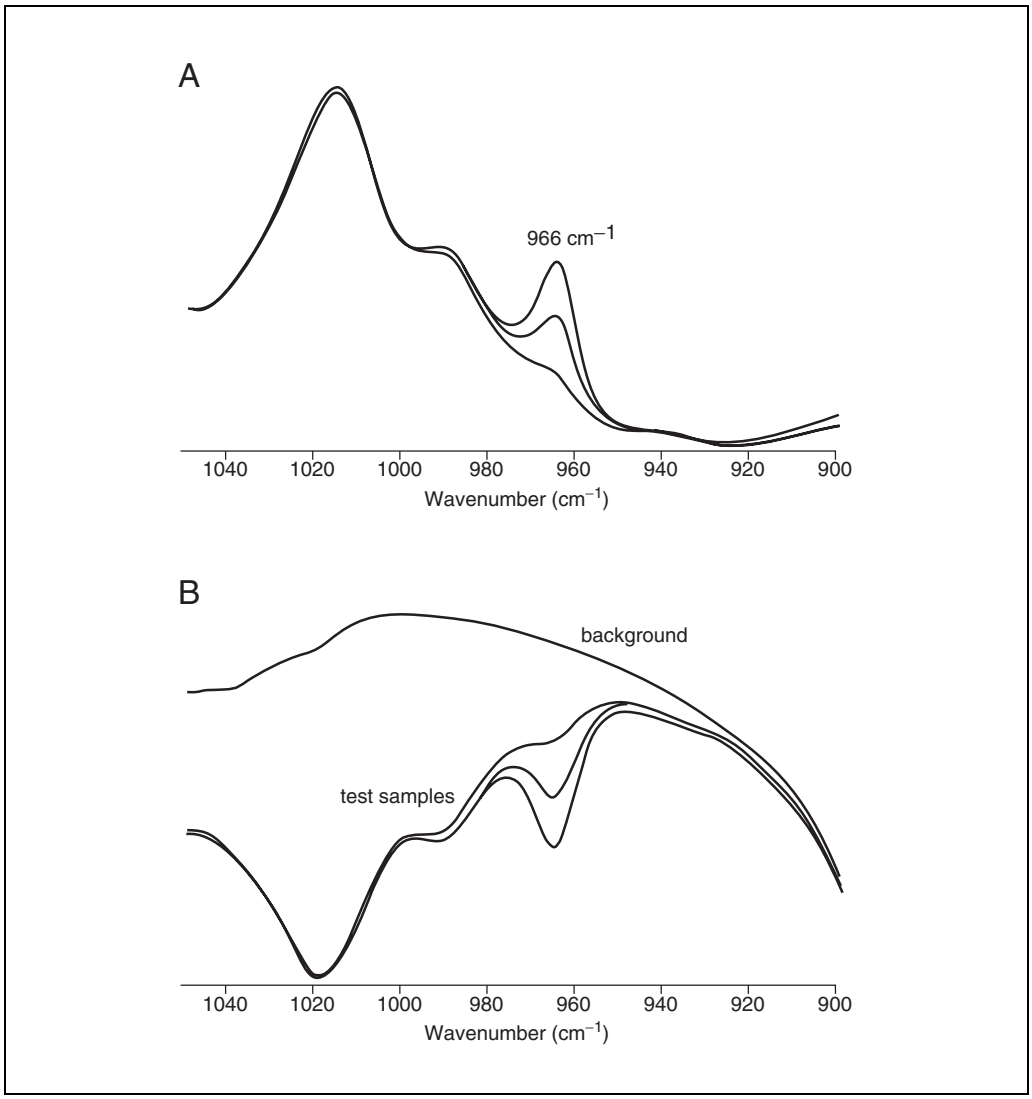

Figure D1.7.2 (**A**) Absorption spectra for carbon disulfide (CS_2) solutions of *trans* fat (test samples). (**B**) Single-beam spectra for CS_2 solvent (background) and CS_2 solutions of *trans* fat (test samples). Reprinted from Mossoba et al. (1996) with permission from the American Oil Chemists' Society.

(Fig. D1.7.1A and D1.7.2A; Mossoba et al., 1996). Traditionally, CS_2 solvent has been used as the reference background material in the vast majority of procedures and official methods (Fig. D1.7.2). When a *trans*-free fat reference background material is used (Fig. D1.7.1B) instead of CS_2, the elevated and sloping baseline of the 966 cm^{-1} *trans* band (Fig. D1.7.2A) becomes *horizontal* (Fig. D1.7.1A; Mossoba et al., 1996; AOCS, 1999a; AOAC International, 2000). Therefore, the contributions of the triacylglycerol absorptions that led to the sloping baseline in the first place are removed, and the requirement for converting triacylglycerols to FAMEs is eliminated. Having a horizontal baseline minimizes the uncertainty in the measurement of the 966 cm^{-1} *trans* band area at all *trans* levels, and improves both precision and accuracy. This approach of using an appropriate *trans*-free reference background material is an integral part of this internal reflection infrared method.

A Fourier-transform infrared (FTIR) spectrometer (Reedy and Mossoba, 1999) consists of a source of continuous infrared radiation that emits light from a high temperature element, an interferometer, and a detector. The interferometer allows the detection of all the component wavelengths of the mid-infrared region (4000 to 600 cm^{-1}) simultaneously. When a test sample (such as a *trans* fat solution) is placed between the beam splitter and the detector, it selectively absorbs infrared energy. Changes in the energy reaching the detector as a function of time yield an interferogram, the raw infrared spectrum. When the interferogram is converted from the time to the frequency domain by the mathematical Fourier transformation, a single-beam spectrum (Fig. D1.7.1B) is obtained. The single-beam spectrum of a "test sample" is the emittance profile of the infrared source as well as the absorption bands of all infrared-absorbing material in the path of the infrared beam, namely the test sample, atmospheric water vapor, and CO_2. A "background" (such as a solvent or a *trans*-free fat solution) single-beam spectrum is also measured. To observe the conventional transmission (or absorption) spectrum of a test sample (Fig. D1.7.2A), the single-beam spectrum of the test sample is digitally "ratioed" against the single-beam spectrum of the reference background.

When melted fat or oil is placed on the surface of an ATR crystal like diamond, the infrared light penetrates a distance of only a few micrometers into the test sample, when the conditions of total internal reflection apply (Harrick, 1967; Mirabella, 1992; Ismail et al., 1998). These conditions occur when light traveling in a transparent medium of high refractive index (η_1), e.g., diamond or ZnSe, strikes the interface between this medium and another transparent medium of lower refractive index (η_2), e.g., air or melted *trans* fat, at an angle of incidence (θ) exceeding the critical angle (θ_c) defined by:

$$\theta_c = \sin^{-1}\left(\frac{\eta_2}{\eta_1}\right)$$

Normally, light is partially transmitted and partially reflected. However, under these conditions it is not transmitted, but totally reflected inside the crystal. Moreover, as the light bounces (one or more times) inside the crystal, a so-called evanescent wave also propagates away from the surface of the crystal through the test sample (melted *trans* fat; Mossoba et al., 1996, 2001b; Adam et al., 2000). At the surface of the crystal, the intensity of this wave decays exponentially with distance. It is also attenuated by the absorption of infrared light by the melted *trans* fat. The depth of penetration (d_p) of the infrared light into the test sample is a trivial distance. It typically varies between 1 and 4 μm and depends on θ, η_2, η_1, and the wavelength (λ) as given by the relation:

$$\frac{\lambda}{2\pi\eta_1\sqrt{\sin^2(\theta)-(\eta_2/\eta_1)}}$$

As a result, the depth of penetration, or effective pathlength, will be higher the greater λ or the smaller the frequency. Therefore, an interferogram (raw infrared spectrum) is a measure of the attenuation of a *trans* fat test sample of the totally internally reflected infrared light. The interferogram of a reference background material (*trans*-free fat) is similarly measured. These are subsequently used to obtain an absorption spectrum.

Capillary gas chromatography

Capillary gas chromatography is an alternative method to measure *trans* fats as FAMEs (AOAC 996.06 and AOCS Ce 1f-96), particularly for lower concentrations (~0.5%). GC official method AOAC 996.06 (AOAC, 2001) is appropriate for the determination of fat in food products, while AOCS Ce 1f-96 (AOCS, 1996) is applicable to the determination of *cis* and *trans* fatty acids in hydrogenated and re-

fined vegetable oils and fats. Using a single capillary GC analysis, AOCS Ce 1f-96 provides qualitative and quantitative determinations for *trans* fatty acid isomers, as well as saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). A successful GC determination of total *trans* FAME composition depends on the experimental conditions dictated by the method used, as well as sound judgment by the analyst to correctly identify peaks attributed to *trans* FAME and their positional isomers. These GC methods for the determination of *trans* FAME describe separations that require long capillary columns with highly polar stationary phases. Unfortunately, partial or complete overlap of many peaks belonging to the two different groups of *trans* and *cis* geometric isomers occurs. This is because the retention time range for late-eluting *trans* 18:1 positional isomers starting at Δ12 is the same as that for the *cis* 18:1 positional isomers, Δ6-Δ14. In addition, *cis/trans* C18 methylene- and non-methylene-interrupted fatty acids retention time range overlaps with that of the *cis*-18:1 positional isomers. Elimination of GC peak overlap requires prior separation of the *cis* and *trans* 18:1 geometric isomers by silver ion–thin layer chromatography (Buchgraber and Ulberth, 2001). A detailed discussion of GC methodologies was recently published in a monograph (Mossoba et al., 2003).

Conjugated linoleic acid (CLA)

Another important dietary source of *trans* fat is conjugated linoleic acid, a class of compounds collectively known as CLA. Many CLA isomers contain conjugated *cis/trans* and *trans/trans* double bonds. Interest in CLA research has increased significantly in the past few years because several *cis/trans* CLA isomers have been reported to exhibit different beneficial physiological effects in animal studies (Yurawecz et al., 1999). The reader is referred to a collection of analytical papers published in a dossier (Mossoba, 2001, and references therein) that details several chromatographic and spectroscopic techniques and procedures that have been successfully applied to CLA analysis.

Critical Parameters and Troubleshooting

Trans-free reference fat

The *trans*-free reference fat should be a material containing either a negligible quantity of *trans* fat, such as a cold-pressed vegetable oil, or a standard (such as triolein) with no *trans* fat. Ideally, the *trans*-free reference fat should resemble the composition of the fat matrix being determined. Different food products contain different types of hard fats or liquid oils, and the nature of the *trans*-free fat or oil should be based closely on the matrix (e.g., vegetable oil, dairy fat, or fish oil) being analyzed. For example, a cold-pressed soybean oil would be an appropriate *trans*-free reference oil for a matrix that consists of partially hydrogenated soybean oil. The analyst will have to make an educated decision when blends of different oils (e.g., soybean, rapeseed, cottonseed, coconut, palm, or partially hydrogenated oils) are being investigated.

ATR crystals

In recent years, more efficient ATR accessories that are suitable for quantitative analyses were developed. In particular, small diamond ATR crystals that have a capacity of ~1 µl have been successfully tested and are recommended for *trans* fat analysis.

ATR sampling

It is essential to ensure that the test portion of the fat being analyzed *completely* covers the horizontal surface of the ATR crystal for the quantitative determination to succeed. In some cases, after placing a fat test portion on the ATR crystal, the melted or liquid fat beads up and partially rolls off the surface of the ATR crystal. The only recourse the analyst would have is to try again.

Cleaning the ATR crystal

Thoroughly wiping the horizontal surface of the ATR crystal with low-lint paper has been the method of choice for cleaning without the use of any solvent. However, to ensure the complete removal of a test sample the analyst should also apply the subsequent test portion and clean the crystal once again. Additionally, the analyst could make an infrared measurement after cleaning the crystal; the absence of a spectrum would confirm that the crystal is clean.

ATR cell

The attenuated total reflection (ATR) infrared cell should be equipped with a single bounce element made of zinc selenide (ZnSe), diamond, or equivalent material, with capacity of 50 µL or less. It must be capable of maintaining a constant temperature of 65° ± 2°C.

FTIR spectrometer

The FTIR spectrometer should be equipped with a deuterium triglyceride sulfate (DTGS) detector capable of making measurements at 4 cm^{-1} resolution in the spectral range covering 1050 to 900 cm^{-1}. Its performance must meet the following criteria. In the absence of a test sample, a 1-min data collection at 4 cm^{-1} resolution must yield, between 1050 and 900 cm^{-1}, a peak-to-peak noise level of <0.0005 AU. The 966 cm^{-1} for a 1% TE standard must yield a signal-to-noise (S/N) ratio of >10:1.

Anticipated Results

All the points used to generate a standard calibration curve (described by a linear regression equation) are expected fall on a straight line. This is due to the fact that the thickness of a test portion is inherently precise with ATR (see Background Information). If a point does not fall on the resulting straight line then the corresponding standard must be discarded and accurately weighed again.

Time Considerations

ATR-FTIR measurements require ~2 min per test sample (Adam et al., 2000; Mossoba et al., 2001b). One sample can be analyzed in 5 min, or 12 samples can be analyzed in 1 hr; analysis includes ATR-FTIR measurement and calculation of *trans* level. The new ATR-FTIR official method (AOCS, 1999a; AOAC International, 2000) can rapidly and conveniently be applied to the determination of the total content of *trans* fat for the vast majority of food lipids.

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Measurement of Primary Lipid Oxidation Products

Lipids are important macromolecules in food. A food product's nutritional value as well as its flavor, texture, general palatability, and storage stability are affected by lipids. Therefore, both physical and chemical criteria are needed by the food processor to assess or monitor the quality of fats and oils. The basic characteristics of certain food items, such as edible oils, will be dependent upon their source. Variation from these norms can be ascertained before the oils are used in other foodstuffs. In effect, knowledge of the quality of the lipid before shipping the product to market, or use in fabricated foods, is of economic importance to the processor.

Lipids are susceptible to oxidation and, as such, analytical protocols are required to measure their quality. Autoxidation is one of the chief processes by which lipids degrade. During the early stages of this autocatalytic free-radical chain reaction, the positions of double bonds in unsaturated fatty acids are changed and hydroperoxides are produced. These alterations can be monitored by simple analytical protocols. Spectrophotometric, iodometric, and colorimetric methods for measuring the early stages of lipid oxidation are described in this unit. In Basic Protocol 1, UV absorbance, measured at 233 and 268 nm, is employed to monitor the formation of conjugated dienes (CDs) and trienes (CTs) of polyunsaturated fatty acids (PUFAs), respectively. Increasing absorption values are an indication that oxidation is proceeding. This physical method is performed on oils directly or on lipid extracts, and is a nondestructive assay. For CD formation to occur, lipid samples that contain unsaturated fatty acids with at least two double bonds are required. The most important lipid of this class in foods is linoleic acid (18:2 ω6). Likewise, unsaturated fatty acid moieties with more than two double bonds are a prerequisite for CT formation. Due to the sensitivity of this technique, only small quantities of lipid are needed, especially when it is suspected that oxidation has taken place.

The determination of peroxide value (PV) by an iodometric titration is described in Basic Protocol 2. Iodine is liberated by hydroperoxides in the oil in the presence of excess iodide in a stoichiometric ratio. The amount of iodine present is determined by titration with a standard sodium thiosulfate solution using a starch indicator, thereby reflecting how much peroxide is present in the oil or lipid extract.

Another approach for the determination of PVs, as described in the Alternate Protocol, is a spectrophotometric method based on the ability of peroxides to oxidize iron(II) to iron(III). The ferric ion forms a complex with xylenol orange, whose concentration can be determined spectrophotometrically. The ferrous oxidation/xylenol orange (FOX) method is very rapid, requires little sample, and can determine PVs as low as 0.1 meq active oxygen/kg oil.

CAUTION: Chemical substances used in this unit require special handling; see *APPENDIX 2B* for guidelines.

DETERMINATION OF CONJUGATED DIENES AND TRIENES

BASIC PROTOCOL 1

This protocol can be used to determine the change in conjugated diene (CD) and triene (CT) formation during storage of an oil as it oxidizes. This is assessed by measuring the change in absorbance at a fixed wavelength in the UV region for a constant mass of sample. An increase in absorbance suggests that the sample is oxidizing. Two methods of presenting this measurement are described for CDs: as a CD value, expressed in μmol CD/g sample, and as a CD extinction value. The extinction coefficient for CTs is currently unknown. Thus, only a CT extinction value can be calculated.

Contributed by Ronald B. Pegg

Current Protocols in Food Analytical Chemistry (2001) D2.1.1-D2.1.15

Materials

Oil or lipid extract sample (*UNIT D1.1*)
2,2,4-trimethylpentane (isooctane), ACS spectrophotometric grade (e.g., Fisher Scientific)

25-ml volumetric flask
Spectrophotometer with ultraviolet (UV) lamp
Quartz cuvettes, 1.00 ± 0.01–cm path length

1. Accurately weigh (and record the weight of) 0.01 to 0.03 g oil or lipid extract sample into a 25-ml volumetric flask.

   Each sample should be assayed at least in duplicate.

2. Dissolve sample in 2,2,4-trimethylpentane and bring to volume. Mix solution thoroughly.

   An ultrasonic bath can be used to aid in sample dissolution.

3. Turn on a spectrophotometer and set the wavelength to 233 nm for CD measurements. Allow instrument to warm up ≥30 min before taking any readings.

4. Zero spectrophotometer with a solvent blank (i.e., 2,2,4-trimethylpentane) using a quartz cuvette.

5. Measure absorbance of the dissolved oil sample using a quartz cuvette.

   If the A_{233} of the sample is >1, the sample should be further diluted in the same solvent and measured again.

6. Repeat absorbance measurements at 268 nm for CT determination.

7. Calculate the CD value using the following equations:

   $c_{CD} = A_{233}/(\varepsilon \times l)$

   CD value = $[c_{CD} \times (2.5 \times 10^4)]/W$

   where c_{CD} is the CD concentration in mmol/ml (i.e., the molar concentration), A_{233} is the absorbance of the lipid solution at 233 nm, ε is the molar absorptivity (i.e., the extinction coefficient) of linoleic acid hydroperoxide ($2.525 \times 10^4\ M^{-1} \cdot cm^{-1}$), l is the path length of the cuvette in cm (1 cm), 2.5×10^4 is a factor that encompasses the volume of 2,2,4-trimethylpentane (25 ml) used to dissolve the oil sample as well as a unit conversion (1000 µmol/mmol) so that the content of CDs can be expressed in µmol, and W is the weight of the sample in g.

   The value for ε is only approximate here, as the extinction coefficient was based on iodometric PV determinations of an ethanolic solution of linoleic acid hydroperoxide (O'Brien, 1969).

8. Calculate CD and CT values from their respective absorbances, but express the results as extinction values, using the following equation:

$$E^{1\%}_{1\ cm} = \frac{A_\lambda}{(c_L \times l)}$$

   where E is the extinction value, A_λ is the absorbance measured at either 233 nm (for CDs) or 268 nm (for CTs), c_L represents the concentration of the lipid solution in g/100 ml, and l is the path length of the cuvette in cm.

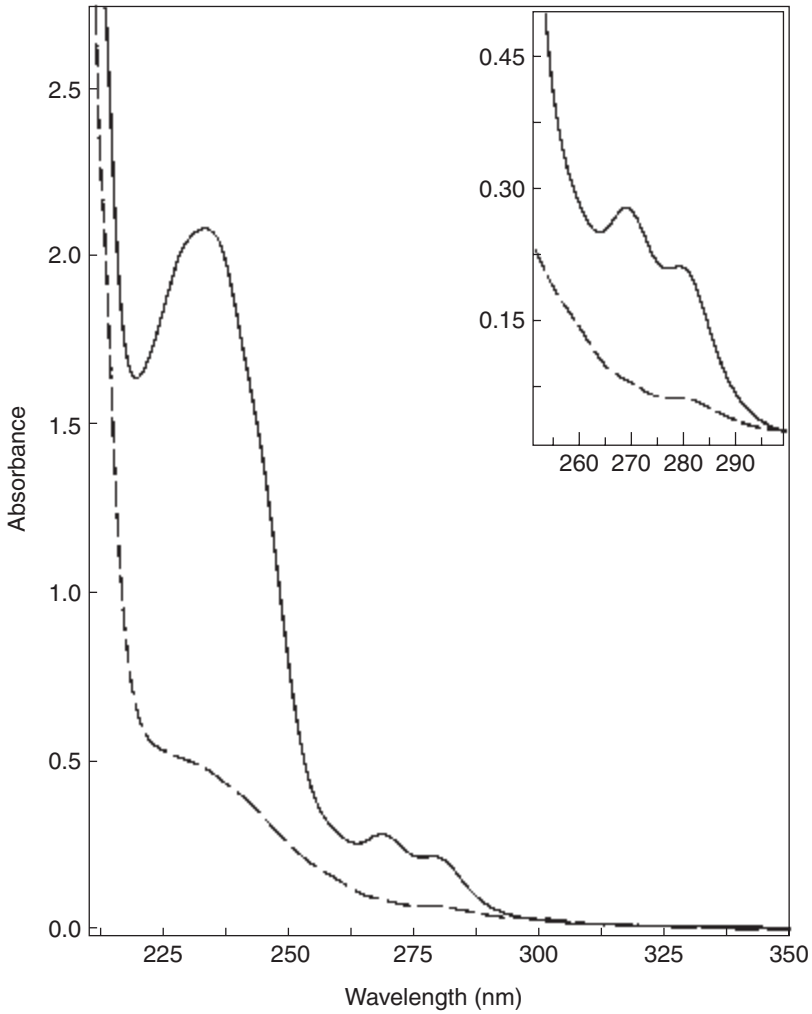

Figure D2.1.1 UV absorbance of sunflower oil samples dissolved in 2,2,4-trimethylpentane. An unoxidized sample (dashed line) and an oxidized sample (solid line) showing the characteristic absorbance peak for conjugated dienes (CDs) at 233 nm as well as peaks at 268 and 278 nm corresponding to conjugated trienes (CTs). The plot in the upper right corner shows an enlarged view of the absorbance curve in this region.

In the scientific literature, extinction values are often used as a measure for the CDs and CTs in oxidized oil samples (Ong, 1980; Brown and Snyder, 1982; Wanasundara and Shahidi, 1994a).

For a sample calculation, a 0.0118-g sample of oxidized sunflower oil was assayed for CD formation (see Figure D2.1.1 for a typical UV-absorption spectrum). The sample was dissolved in 25 ml of 2,2,4-trimethylpentane. The spectrophotometer was zeroed, and the absorbance of the oil sample at 233 nm was measured as 0.9056. Therefore, c_{CD} *= 0.9056/(2.525* $\times$ *10*^{4} *M*$^{-1}$*·cm*$^{-1}$ *× 1 cm) = 3.58* $\times$ *10*$^{-5}$ *mmol/ml, and the CD value = (3.58* $\times$ *10*$^{-5}$ *mmol/ml* $\times$ *2.5* $\times$ *10*^{4}*)/0.0118 g = 76 μmol/g. The result can also be expressed as an extinction value:* $E^{1\%}_{1cm}$ *= 0.9056/(0.0472 g/100 ml* $\times$ *1 cm) = 19.2.*

BASIC PROTOCOL 2

DETERMINATION OF PEROXIDE VALUE BY IODOMETRIC TITRATION

The peroxide value (PV) of an oil or fat is defined as the quantity of peroxide oxygen present in the sample. This classical iodometric method is a volumetric analysis based on the titration of iodine released from potassium iodide by peroxides in a biphasic system using a standardized thiosulfate solution as the titrant and a starch solution as the indicator (see Background Information, discussion of peroxide value). This method will detect all substances that oxidize potassium iodide under the acidic conditions of the test, therefore the purity of the reagents is critical.

Confusion may exist as to how PVs are expressed. Hendrikse et al. (1994) define PV as mmol active oxygen (i.e., peroxide)/2 kg sample. However, other expressions for PV include meq active oxygen/kg sample, mmol active oxygen/kg sample (i.e., the Lea value; Lea, 1946), or mg active oxygen/kg sample. These units can be converted to mmol active oxygen/2 kg sample by multiplying by the factors 1, 0.5, and 8, respectively.

Materials

Oil or lipid extract sample (*UNIT D1.1*)
3:2 (v/v) acetic acid/chloroform solution (use ACS-grade reagents; store <2 weeks at room temperature)
Saturated potassium iodide solution, tested for stability (see recipe)
~0.1 N sodium thiosulfate solution, standardized (see Support Protocol)
1% (w/v) starch indicator solution, tested for stability (see recipe)

250-ml glass-stoppered Ehrlenmeyer flasks
10- or 25-ml graduated class-A glass buret

NOTE: Deaerate all solutions before beginning protocol.

1. Accurately weigh (and record the weight of) 5.00 ± 0.05 g oil or lipid extract sample into a 250-ml glass-stoppered Ehrlenmeyer flask.

   If oxidation is suspected, less sample should be used for the analysis (Table D2.1.1).

   Each sample should be assayed at least in duplicate.

2. Add 30 ml of 3:2 acetic acid/chloroform solution and swirl flask until oil is dissolved.

   CAUTION: *This step should be carried out in a fume hood to avoid inhalation of the vapors. Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Contact with skin should be avoided. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal.*

3. Use a micropipettor to add 0.5 ml saturated potassium iodide solution to flask.

4. Allow solution to stand exactly 1 min with occasional shaking and then add 30 ml distilled water.

Table D2.1.1 Sample Size for Idometric PV Determination

Expected PV (meq active oxygen/kg sample)	Weight of sample to use (g)
0-2	5
2-10	2
10-25	1
25-50	0.5
50-100	0.3

5. Using a 10- or 25-ml graduated class-A glass buret, gradually add standardized ~0.1 N sodium thiosulfate to flask to titrate solution until the yellow color has almost disappeared. Shake constantly and vigorously.

   A magnetic stirrer and stir-bar can be used to provide effective mixing and to watch the color change during the titration more easily.

   If the expected peroxide content in the sample is low, a standardized ~0.05 N or ~0.01 N sodium thiosulfate solution should be used for the titration.

   These solutions can be prepared by accurately pipetting 500 ml or 100 ml of ~0.1 N sodium thiosulfate, respectively, into a 1000-ml volumetric flask and then accurately diluting to volume with recently boiled distilled water.

6. Use a micropipettor to add ~0.5 ml of 1% starch indicator solution.

7. Continue titration, shaking flask vigorously near the endpoint to liberate all iodine from the chloroform layer. Add sodium thiosulfate solution dropwise until the violet color disappears. Record the total added sodium thiosulfate volume.

8. Conduct a blank determination of the reagents by following steps 2 to 7 (i.e., without the addition of oil or lipid extract sample).

   The blank titration must not exceed 0.1 ml sodium thiosulfate solution. If a 0.1-ml volume is exceeded, fresh reagents should be prepared and the new thiosulfate solution standardized. If the problem persists, the purity of the chemicals employed should be examined.

   Measurement of a blank need only be carried out once for each experimental day.

9. Calculate the PV using the equation:

   $$PV = [(S - B) \times N \times 1000]/W$$

   where S is the volume (ml) of sodium thiosulfate required to titrate the sample, B is the volume (ml) of sodium thiosulfate required for the blank, N is the calculated normality of the standardized sodium thiosulfate solution (see Support Protocol), and W is the weight of the sample (g).

   From this equation, the PV is expressed as meq active oxygen/kg sample and is equal to mmol active oxygen/2 kg sample.

PREPARATION AND STANDARDIZATION OF SODIUM THIOSULFATE SOLUTION

SUPPORT PROTOCOL

The exact normality of the sodium thiosulfate solution (~0.1 N $Na_2S_2O_3$) must be calculated before iodometric PV determinations can be made. A properly made thiosulfate solution will require only occasional restandardization.

Materials

Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$, formula wt. = 248.17), ACS grade
Sodium carbonate (Na_2CO_3)
Potassium iodate (KIO_3) for primary standard
Potassium iodide (KI), iodate free, ACS grade
6.0 M HCl (*APPENDIX 2A*)
1% (w/v) starch indicator solution, tested for stability (see recipe)

Clean stoppered bottle
Oven, 110°C

Prepare solution

1. Boil ~1 liter water ≥5 min and allow to cool.
2. Add ~25 g $Na_2S_2O_3 \cdot 5H_2O$ and 0.1 g Na_2CO_3. Stir until all crystals are dissolved and transfer to a clean stoppered bottle.

   The solution will be ~0.1 N. It can be stored at this point for up to 4 weeks in the dark at room temperature.

Standardize solution

3. Dry primary standard–grade potassium KIO_3 ≥1 hr in an oven at 110°C and then cool in a desiccator.
4. Using an analytical balance, accurately weigh (and record the weight of) 0.10 to 0.15 g KIO_3 into a 250-ml Ehrlenmeyer flask.
5. Dissolve crystals in 75 ml water and add ~2 g iodate-free KI.
6. Add 2 ml of 6.0 M HCl and titrate immediately with sodium thiosulfate solution until the color of the solution becomes pale yellow. Shake constantly and vigorously.
7. Use a micropipettor to add 0.5 ml of 1% starch indicator solution.
8. Continue titration, adding sodium thiosulfate dropwise until the violet color disappears. Record the total added sodium thiosulfate volume.
9. Repeat the experiment twice more, and carry out one titration without KIO_3 (i.e., a blank determination).
10. Calculate the normality of the sodium thiosulfate solution using the equation:

    $N = (28.037 \times W)/(S - B)$

    where N is the normality of the sodium thiosulfate solution, W is the weight of the KIO_3 (g), S is the volume (ml) of sodium thiosulfate required to titrate the sample, and B is the volume (ml) of sodium thiosulfate required for the blank.

ALTERNATE PROTOCOL

DETERMINATION OF PEROXIDE VALUE BY MEASUREMENT OF IRON OXIDATION

The ferrous oxidation/xylenol orange (FOX) method is based on the ability of lipid peroxides to oxidize ferrous ions at low pH. The resulting oxidation is quantitated by using a dye that complexes with the generated ferric ions to produce a color that can be measured spectrophotometrically. Peroxide values (PVs) as low as 0.1 meq active oxygen/kg sample can be determined with this method, providing a distinct advantage over iodometric titration.

Materials

Oil or lipid extract sample (*UNIT D1.1*)
7:3 (v/v) chloroform/methanol solution (use ACS-grade reagents; store 2 weeks at room temperature)
10 mM 3,3′-bis(*N*,*N*-di[carboxymethyl]aminomethyl)-*o*-cresolsulfonephthalein (xylenol orange; e.g., Aldrich; store <1 month at room temperature)
Iron(II) chloride solution (see recipe)
Iron(III) chloride standard solution (10 µg/ml $FeCl_3$; see recipe)

16 × 125–mm borosilicate glass tubes
Spectrophotometer
Glass cuvettes, 1.00 ± 0.01–cm path length

NOTE: The formula weight for xylenol orange sodium salt will vary depending on whether the four acid moieties are protonated or exist as sodium analogs. Therefore, the formula weight will be either 694.6, 716.6, 738.6, 760.6, or 782.6.

NOTE: Deaerate all solutions before beginning protocol.

1. Accurately weigh (and record the weight of) 0.01 to 0.30 g oil or lipid extract sample into a 16 ×125–mm borosilicate glass tube.

   If oxidation is suspected, less sample should be used for the analysis.

2. Add 9.9 ml of 7:3 chloroform/methanol solution. Vortex sample 2 to 4 sec.

   CAUTION: *This step should be carried out in a fume hood to avoid inhalation of the vapors. Chloroform is a known carcinogen. It is toxic by inhalation and has anesthetic properties. Contact with skin should be avoided. Prolonged inhalation or ingestion can lead to liver and kidney damage and may be fatal.*

3. Turn on a spectrophotometer and set wavelength to 560 nm. Allow instrument to warm up ≥30 min before taking any readings.

4. Zero spectrophotometer with a solvent (i.e., chloroform/methanol solution) blank using a glass cuvette.

5. Add 50 µl of 10 mM xylenol orange solution to sample, vortex 2 to 4 sec, and then add 50 µl iron(II) chloride solution and vortex again.

   The final volume in the test tube should be 10 ml.

6. Allow solution to stand exactly 5 min at room temperature and then determine its absorbance at 560 nm.

   Be sure to adhere strictly to the 5-min incubation period.

7. Construct a standard curve by repeating steps 1 to 6 without using oil or lipid extract sample. Instead of the sample, add to a series of 16 × 125–mm borosilicate glass tubes varying aliquots of an iron(III)–chloride standard solution (10 µg/ml), 50 µl of 10 mM xylenol orange solution, and enough 7:3 (v/v) chloroform/methanol solution to a final volume of 10 ml.

   Appropriate volumes of the iron(III) chloride standard solution should range from 0 to 2 ml.

   The absorbance reading for the test tube with only xylenol orange and the 7:3 chloroform/methanol solution represents the absorbance of the blank.

8. Plot absorbance (A_{560}) of the standards on the *y* axis versus µg Fe^{3+} on the *x* axis and fit with a regression line of the form $y = mx + b$, where *m* is the slope of the line and *b* is the *y* intercept.

   Shantha and Decker (1994) reported that the curve of A_{560} versus Fe^{3+} concentration for the modified FOX method is not linear for wide ranges of Fe^{3+}. Therefore as stated above, only the range from 5 to 20 µg Fe^{3+} should be used to construct the calibration curve.

9. Calculate PV, expressed as meq active oxygen/kg sample, using the following equation:

   $$PV = [(A_S - A_B) \times m_i]/(W \times 55.84 \times 2)$$

   where A_S is the absorbance of the sample, A_B is the absorbance of the blank, m_i is the inverse of the slope (i.e., 1/slope) obtained in step 8 (a typical value is 25.5; Shantha and Decker, 1994), *W* is the weight of the sample (g), and 55.84 is the atomic weight

of iron (µg/µmol). The factors to convert g to kg and µmol to mmol cancel out one another, but a value of 2 is necessary in the denominator of the equation to express the PV as meq active oxygen (i.e., peroxide) instead of meq. of oxygen.

REAGENTS AND SOLUTIONS

Use ACS-grade chemicals unless otherwise specified and deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Iron(II) chloride solution

Dissolve 0.5 g $FeSO_4 \cdot 7H_2O$ in 50 ml water. In a separate flask, dissolve 0.4 g $BaCl_2 \cdot 2H_2O$ in 50 ml water. Slowly add the barium chloride solution to the iron(II) sulfate solution with constant stirring, followed by 2 ml of 10 M HCl. Filter off barium sulfate precipitate by gravity using Whatman no. 1 filter paper. Store <1 month in a brown bottle in the dark at room temperature.

Iron(III) chloride standard solution

Dissolve 0.5 g $FeCl_3$ in 50 ml of 10 M HCl and add 1 to 2 ml of 30% (v/v) H_2O_2. Boil solution ~5 min to remove excess H_2O_2 and allow to cool to room temperature. Dilute to 500 ml with water and transfer a 1-ml aliquot to a 100-ml volumetric flask. Dilute to mark with 7:3 (v/v) chloroform/methanol solution (final 10 µg/ml $FeCl_3$). Store up to 2 weeks at room temperature.

Chloroform/methanol solution should be prepared fresh every 2 weeks.

Potassium iodide solution, saturated

Boil some water ≥5 min and allow to cool. To a portion, add enough potassium iodide (KI, iodate free, ACS grade) to ensure a saturated solution (~10.0 g KI in 6.0 ml water). Prepare the night before use, store overnight in the dark, and make sure the solution remains saturated as indicated by the presence of undissolved crystals.

To test stability, dilute 0.5 ml saturated potassium iodide solution in 30 ml of 3:2 (v/v) acetic acid/chloroform and then add 2 drops of 1% starch indicator solution (see recipe). If a violet color forms that requires more than 1 drop of 0.1 N sodium thiosulfate solution to discharge, discard the iodide solution and prepare a fresh one.

Acetic acid/chloroform solution should be prepared fresh every 2 weeks.

The stability of the saturated KI solution should be tested daily.

Starch indicator solution, 1 % (w/v)

Make a paste by rubbing ~1 g soluble starch in ~30 ml water. Transfer mixture to ~80 ml boiling water and heat until a clear solution results. Cool and store in stoppered bottle. Store at 4° to 10°C for 2 to 3 weeks. Prepare fresh indicator when the end point of the titration from violet to colorless fails to be sharp.

To test sensitivity, place 5 ml starch solution in 100 ml water and add 0.05 ml freshly prepared 0.1 N KI solution and one drop of a 50-ppm chlorine solution made by diluting 1 ml commercial 5% sodium hypochlorite (NaOCl) to 1000 ml. The deep violet color produced must be discharged by 0.05 ml of 0.1 N sodium thiosulfate (see Support Protocol).

Salicylic acid (1.25 g/l) may be added to preserve the indicator.

Potato starch for iodometry is recommended, because this starch produces a deep violet color in the presence of the iodonium ion. On the other hand, soluble starch is not recommended because a consistent deep violet color may not be developed when some soluble starches interact with the iodonium ion.

COMMENTARY

Background Information

Lipids are susceptible to oxidation and, therefore, analytical protocols are required to measure their quality. Not all lipids have the same degree of susceptibility to oxidation. Many factors are responsible for a lipid's tendency to oxidize, including the presence of catalysts, oxidative enzymes, radiation, and a lipid-air interface, as well as the oxygen partial pressure, the incorporation of oxygen into the product, and the presence of metal ions. The most important factor is the degree of unsaturation of the lipid itself. The majority of a food product's polyunsaturated fatty acids (PUFAs) are generally contained in phospholipids, which are consequently more prone to autoxidation than the triacylglycerol fraction.

The primary products from autoxidation are hydroperoxides, which are often simply referred to as peroxides. Peroxides are odorless and colorless, but are labile species that can undergo both enzymatic and nonenzymatic degradation to produce a complex array of secondary products such as aliphatic aldehydes, alcohols, ketones, and hydrocarbons. Many of these secondary oxidation products are odiferous and impart detrimental sensory attributes to the food product in question. Being able to monitor and "semi-quantitate" the development of peroxides by objective means (e.g., PV determination) over time is important for food scientists who want to characterize the quality of an oil or a lipid-containing food product, even though the peroxides themselves are not directly related to the actual sensory quality of the product tested.

Measuring the content of primary oxidation products is limited due to the transitory nature of peroxides. Yet, their presence may indicate a potential for later formation of sensorially objectionable compounds. The peroxide content increases only when the rate of peroxide formation exceeds that of its destruction. In cases where peroxide breakdown is as fast as or faster than peroxide formation, monitoring lipid peroxides is not a good indicator of oxidation. This can occur in frying oils and sometimes in meat products, particularly in cooked meats where iron is very active and peroxide breakdown is quite rapid. Because the acceptability of an oil or lipid-containing food product depends on the degree to which oxidation has progressed, the simultaneous detection of primary and secondary lipid oxidation products helps to better characterize lipid quality. It is important to remember that there is no ideal chemical method that correlates well with changes in organoleptic properties of oxidized lipids throughout the entire course of autoxidation. For this reason, the formation and degradation of both primary and secondary products of lipid oxidation should be monitored over time.

Conjugated dienes and trienes

In organic chemistry, the term conjugated diene (CD) refers to two double bonds separated by a single bond. This structure is unusual for PUFAs, as they have a divinylmethane structure (i.e., they are nonconjugated). Therefore, it is generally accepted that the presence of CDs (i.e., double-bond migration leading to conjugation) in lipids indicates that autoxidation of fatty-acid moieties has occurred (Corongiu and Banni, 1994). The divinylmethane structure of PUFAs makes them susceptible to hydrogen abstraction by free-radical attack. The PUFA becomes a free-radical intermediate, which then rearranges the double bond to form a CD or polyene (Figure D2.1.2B). Attack by molecular oxygen results in a lipid peroxyl radical, which can abstract a hydrogen atom from an adjacent lipid molecule to form a lipid hydroperoxide (Figure D2.1.2C) or endoperoxide.

The CD moiety is a strong UV-absorbing chromophore that can be detected spectrophotometrically. When present in fatty acids, the CD moiety shows an absorption in the UV region at 233 nm and stands out as a distinct peak. When PUFAs containing three or more double bonds (e.g., linolenic acid) undergo oxidation, the conjugation of CD moieties can be extended to include another double bond resulting in the formation of a conjugated triene (CT). CTs, which may exist naturally in certain fats (e.g., tung oil) or are formed during industrial treatment (e.g., on bleaching by bleaching earths), absorb radiation in the UV region like CDs, but show three signature absorption bands. The principal peak is at ~268 nm (IUPAC, 1987b). There is a secondary peak at 278 nm and minima adjacent to the principal peak at 262 and 274 nm. Therefore, an increase in the absorbance of oils or extracted membrane lipids at these characteristic bands serves as an indicator of lipid oxidation.

Figure D2.1.1 depicts changes in the UV spectrum of a sunflower oil sample after oxidation. The unoxidized oil sample dissolved in

2,2,4-trimethylpentane exhibits no characteristic absorption bands; after oxidation, the 233 nm band, indicative of CDs, is quite distinct. The presence of conjugated trienes is only evident if the oil sample contains fatty acids with more than two double bonds; thus, measurement for CTs is not always necessary. In the case of sunflower oil, the primary and secondary absorption bands for CTs are obvious at 268 and 278 nm, respectively, as are minima at 263 and 275 nm. The upper right corner of Figure D2.1.1 contains an enlargement of the UV spectra for the sunflower oil sample in this region.

The detection of CDs in unsaturated lipids is a sensitive assay, but the magnitudes of changes in absorption are not easily related to the extent of oxidation; the effects upon the numerous unsaturated fatty acids vary in quality and magnitude (Gray, 1978). However, during the early stages of oxidation, the increase in UV absorption due to the formation of CDs and CTs is proportional to the uptake of oxygen and generation of peroxides. For this reason, the content of CDs and CTs can serve as a relative measurement of oxidation (Farmer and Sutton, 1946). The CDs accumulate to a certain percentage in any one oil and then plateau as the formed dienes are involved in additional oxidative reactions, which cause their breakdown (White, 1995).Shahidi et al. (1994) and Wanasundara et al. (1995) found that CD levels, expressed as extinction values, and PVs of edible oils correlated well during oxidation. These authors concluded that the CD technique may be used as an index of stability of lipids in lieu of, or in addition to, PV determinations. In general, the CD and CT methods are faster than PV determinations, are much simpler, require no chemical reagents, do not depend on chemical reactions or color development, and require a smaller sample size. However Corongiu and Banni (1994) noted that detection and quantitation of CDs in mixtures of peroxidized and nonperoxidized lipids, by means of simple UV spectrophotometry, is complicated by the end absorption exhibited by naturally occurring and nonperoxidized lipids as well as by contaminants in lipid extracts. A good case in point is for lipid extracts from muscle food samples. Background absorption in the UV region can obscure the 233 nm band, thus making measurement of CDs impossible. Corongiu and Banni (1994) suggested that derivative spectroscopy, which produces narrower bands and can give information not revealed by simple UV absorption spectra, might resolve some of these issues.

Peroxide value

Autoxidation is a chemical reaction that usually takes place at ambient temperatures between atmospheric oxygen and a lipid substrate. In the presence of an initiator such as light, heat, or metal ions, unsaturated lipids (LH) form carbon-centered alkyl radicals ($L^{\bullet}$):

$$LH \xrightarrow{\text{initiator}} L + H$$

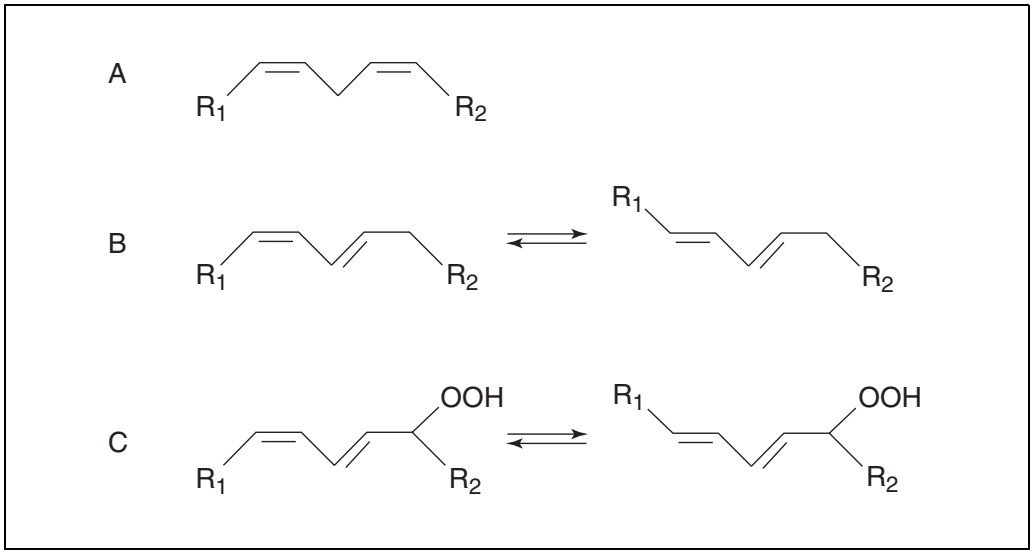

Figure D2.1.2 Different types of conjugated and nonconjugated double bonds in fatty acids. (**A**) A polyunsaturated fatty acid (PUFA). (**B**) A PUFA nonhydroperoxide with a conjugated diene (CD). (**C**) A PUFA hydroperoxide with a CD. R_1 and R_2 indicate the remaining alkyl portions of the PUFA. Reprinted from Corongiu and Banni (1994) with permission from Academic Press.

These radicals react rapidly with molecular oxygen to generate unstable peroxyl radicals (LOO•):

$$L + O_2 \rightarrow LOO$$

that can then abstract a hydrogen atom from another lipid molecule to form a hydroperoxide (LOOH) and a new free radical:

$$LOO + LH \rightarrow LOOH + L$$

This process continually generates lipid free radicals. The formation of nonradical products resulting from the combination of two radical species can terminate this chain reaction or propagation. Alternatively, unsaturated lipids can form hydroperoxides by reacting with singlet oxygen produced by sensitized photooxidation, which is a non-free-radical process.

Peroxides in general (ROOH) can be measured by titrimetric methods based on their oxidation potential to oxidize iodide (I^-) to iodine (I_2):

$$ROOH + 2I^- + 2H^+ \rightarrow ROH + I_2 + H_2O$$

which is then titrated against a standard thiosulfate solution:

$$I_2 + 2Na_2S_2O_3 \xrightarrow{\text{starch}} Na_2S_4O_6 + 2NaI$$

or by colorimetric methods based on their oxidation potential to oxidize iron(II) to iron(III). The ferric ion then reacts with thiocyanate or a dye (i.e., xylenol orange) to form a complex that is measured spectrophotometrically.

The official AOCS (1998; Cd 8-53) and IUPAC (1987a) methods for PV determination are iodometric ones. They are based on the measurement of the iodine liberated from excess KI by the peroxides present in the oil. As oxidation is a dynamic process, the PV is an indication of the amount of peroxides present in a sample at a particular point in time. The results are generally expressed as milliequivalents active oxygen (i.e., peroxide) per kilogram oil (meq/kg). This method is easy and produces consistent results. However, the two principal sources of error in the assay are (1) the absorption of iodine at unsaturated bonds of the fatty material, and (2) the liberation of iodine from potassium iodide by oxygen present in the test sample to be titrated. The latter is often referred to as the oxygen error and leads to high results in the peroxide determination. Other possible sources of error in this method include variation in the weight of sample, the type and grade of solvent used, variation in the reaction conditions such as time and temperature, and the constitution and reactivity of the peroxides being titrated. Although the PV is applicable for following peroxide formation during the early stages of oxidation, it is nevertheless highly empirical and any change in procedure may cause variation in results. In other words, its accuracy is questionable, and the results vary with the specific procedure employed. As peroxides are vulnerable to further reaction, the complete oxidative history of the oil may not be revealed.

After the induction period, the initial rate of peroxide formation is exceeded by its rate of decomposition, but this trend is reversed at later stages; that is, during the course of oxidation, PVs reach a maximum and then decline. Whether a lipid is in the growth or decay section of the peroxide concentration curve will be indicated by monitoring the quantity of peroxides as a function of time (Shahidi and Wanasundara, 1998). Various attempts have been made to correlate PVs with the development of oxidative off-flavors. Good correlations are sometimes obtained, as has been the case with organoleptic flavor scores for a number of commercial fats such as lard, hydrogenated soybean oil, and corn oil. Unfortunately, these are not always found. The amount of oxygen that must be absorbed by the lipid, or the level of peroxides that must be formed to produce noticeable oxidative rancidity, varies with the composition of the oil in question (i.e., those that are more saturated require less oxygen absorption to become rancid), the presence of antioxidants and trace metals, as well as the conditions of oxidation.

The PV is greatly reduced by the refining process used for most vegetable oils. Virgin olive oils are not exposed to such processes and the PVs permitted in these products are considerably higher. Extra-virgin olive oils are permitted PVs ≤20 meq/kg under international standards, whereas pure olive oils, which by definition are blends of virgin and refined olive oils, are required to have PVs <10 meq/kg.

Low PVs cannot be adequately measured by the official methods (AOCS, 1998; IUPAC, 1987a) because of uncertainty with the iodometric titration endpoint; the disappearance of the pale-violet color produced by the iodine and starch reaction is difficult to discern. The test has been modified by replacing the titration step with an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential (Oishi et al., 1992). PVs ranging from 0.06

to 20 meq/kg have been determined by this method. It is essential, of course, during the determination to deaerate all solutions, since the presence of oxygen can lead to further formation of peroxides.

Alternatively, the spectrophotometric determination of ferric ions formed from the oxidation of ferrous ions by peroxides in the presence of xylenol orange, also termed the FOX (ferrous oxidation/xylenol orange) method, has been successfully employed for the determination of lipid hydroperoxides in liposomes and low-density lipoproteins (Gupta, 1973; Jiang et al., 1992). The method is based on the ability of peroxides to oxidize ferrous ions. The resulting oxidation is quantitated by using a compound that complexes with ferric ions to produce a color that can be measured spectrophotometrically. Shantha and Decker (1994) employed a modified FOX technique to measure PVs of cooked beef patties, chicken, butter, and vegetable oil as well as menhaden oil, and compared the data with those obtained by the official methods; the data were similar.

The time to reach a certain PV may be used as an index of oxidative stability for food lipids. The effects of antioxidants and food processing on fats are often monitored in this way. Thus, a longer time period to reach a certain PV is generally indicative of a better antioxidant activity for the additive under examination. However, a low PV represents either early or advanced oxidation; the breakdown of peroxides to secondary oxidation products will result in a decrease in PVs during the storage period. For determination in foodstuff, a major disadvantage to the classical iodometric PV assay is that a 5-g test portion is required; it is sometimes difficult to obtain sufficient quantities of lipid from foods low in fat. Despite its drawbacks, PV determination is one of the most common tests employed to monitor lipid oxidation.

Critical Parameters and Troubleshooting

Conjugated diene and triene determinations

For CD and CT determinations (see Basic Protocol 1), quartz cuvettes must be used, as glass ones will absorb UV radiation. A 1-cm-path-length quartz cuvette is most often employed for spectrophotometric detection; however, quartz cuvettes with shorter path lengths (0.01 to 0.5 cm) are available. The equation relating CD value to absorbance assumes that the cuvette has a path length of 1 cm; when cuvettes of shorter path length are used, the correct value for l must be substituted in the equation. It is also important that the 2,2,4-trimethylpentane be pure and of UV spectrophotometric grade. A check test with 2,2,4-trimethylpentane that the glassware is free from impurities showing absorbance at wavelengths between 200 and 320 nm is often beneficial.

It should be noted that both linoleic and α-linolenic acids form hydroperoxides that absorb UV radiation at 233 nm (i.e., the same wavelength as that of CDs). Furthermore, CDs are formed upon decomposition of hydroperoxides from α-linolenic acid, absorbing at 233 nm, whereas secondary oxidation products, particularly ethylenic diketones and α-unsaturated ketones, show a maximum absorbance at ~268 nm. Carotenoid-containing oils may interfere in the assay by giving higher than expected absorbance values at 233 nm, due to the presence of double bonds in the conjugated structures of carotenoids.

Brown and Snyder (1982) reported that soy oils that had been partially hydrogenated showed higher than expected CD values. This resulted from CD formation during the hydrogenation process. These authors suggested that measurement of CDs is of little use when evaluating the quality of oils or fats that have been hydrogenated.

Peroxide value determinations

It should be stressed again that the classical iodometric method (see Basic Protocol 2) is an empirical one, and therefore any variation in procedure may affect the results. The procedure also fails to adequately measure low PVs because of difficulties encountered in determining the titration endpoint. Although Basic Protocol 2 uses 5 g oil or lipid extract sample, the amount of sample to be weighed depends on the expected PV as shown in Table D2.1.1.

In the case of liquid oils, care should be exercised so that no aeration of the oil occurs before sampling. Solid fats should not be melted beforehand and the sample should be taken from the center of the mass (Hendrikse et al., 1994). It is essential, of course, to deaerate all the solutions used in the protocol, as the presence of oxygen can lead to further formation of peroxides.

Standardization of the thiosulfate solution is important. Various factors can affect its stability. Chief among these are pH, the presence of microorganisms and impurities, the presence of atmospheric oxygen, and exposure to sunlight. Proper attention to detail will yield standard thiosulfate solutions that need only occasional

restandardization. The stability of thiosulfate solutions is at a maximum in the pH range between 9 and 10. Therefore, the addition of a small amount of base such as sodium carbonate, borax, or disodium hydrogen phosphate is recommended to preserve standard solutions of the reagent. The most important single cause of instability can be traced to certain bacteria that metabolize the thiosulfate ion, converting it to sulfite, sulfate, and elemental sulfur. Therefore, it is common practice to impose reasonably sterile conditions during the preparation of standard solutions. Bacterial activity appears to be at a minimum at a pH between 9 and 10, which accounts, at least in part, for the maximum stability of thiosulfate solutions in this range.

The stability of the potassium iodide solution should be tested. Additionally, as starch solutions tend to lose their potency as an indicator due to breakdown of glucose linkages by microorganisms, fresh starch indicator solution should be prepared.

In the modified FOX method (see Alternate Protocol), the 5- min incubation period must be strictly adhered to because the color intensity changes with time. Incubation for an additional 5-min increases the absorption of the blank. Jiang et al. (1992) proposed that the changes in color with time might result from instability of the dye on further oxidation of the sample. The color of the iron-xylenol orange complex changes from reddish to purple as the level of peroxides in the sample increases. This causes a decrease in absorbance at 560 nm. As noted in the protocol itself, the content of Fe^{3+} versus absorbance is not linear over wide concentrations of Fe^{3+}. Therefore, a range of 5 to 20 μg Fe^{3+} should be used when constructing calibration curves (Shantha and Decker, 1994).

Anticipated Results

For oil samples believed to be fresh, low CD, CT, and PV levels are expected. To monitor the oxidation process in refined, bleached, and deodorized canola and soybean oils, Wanasundara et al. (1995) employed Schaal oven test conditions to accelerate the oxidation process. The Schaal oven test at 65°C is considered to be equivalent to one month of storage at ambient temperatures. During 30 days of storage, CD values (expressed as extinction values) ranged from 3.7 to 30 and 4.9 to 52, whereas CT values ranged from 0.8 to 6 and 0.8 to 8 for canola and soybean oils, respectively. It is noteworthy that canola reached a higher level of CDs as compared to soybean oil over a 15-day storage period at 65°C, but after longer storage times, soybean oil showed CD values greater than those of canola oil.

Oishi et al. (1992) compared the results from classical iodometric PV determinations of edible oils and fats to those using a coulometric detector. Results from each technique expressed as meq active oxygen/kg sample, were consistent with one another. Typical results were: sesame oil (4.1), corn oil (8.7), cottonseed oil (14.5), rapeseed oil (33.2), peanut oil (30.5), olive oil (17.0), palm oil (8.9), beef tallow (2.5), and lard (35.0).

To test the efficacy of phenolic compounds against oxidation, Wanasundara and Shahidi (1994b) added a number of flavonoids, butylated hydroxyanisole (BHA), and butylated hy-

Table D2.1.2 PVs of Edible Oils and Fats Determined by the AOAC and Modified FOX Methods[a]

Sample	Storage (days)	PV (meq peroxide/kg sample)	
		AOAC	FOX
Butter	0	ND	0.1
	7	8.3	7.5
	14	12.8	13.1
	~550	215	228
Chicken fat	0	ND	0.3
	7	2.2	2.1
Cooked beef fat	0	ND	0.3
	5	4.3	4.7
	7	2.1	2.5
Vegetable oil	0	0.9	1.3
	5	3.6	4.4

[a]Adapted from Shantha and Decker (1994) with permission from AOAC International. ND, not detected.

droxytoluene (BHT) to refined, bleached, and deodorized canola oil. They stored the oils under Schaal oven test conditions and monitored the progression of oxidation. Over a 13-day period, PV for the untreated sample increased from 0.4 to 159 meq/kg oil, whereas the BHA-treated control increased from 0.4 to 91 and the myricetin-treated sample reached only a value of 28. It should be noted that PVs can range anywhere from 0 to >300 meq active oxygen/kg sample.

The PVs for most fats and oils determined by the modified FOX assay are similar to those obtained by the official AOAC method (see Table D2.1.2); albeit, PVs from the FOX assay were slightly higher in most cases. PVs as high as 200 meq active oxygen/kg oil were determined successfully by the FOX method. However, the great advantage that the FOX assay has over the official iodometric method stems from its sensitivity in determining low peroxide levels. PVs as low as 0.1, 0.3, and 0.3 meq active oxygen/kg sample were determined in fresh butter, cooked chicken fat, and cooked beef fat, respectively.

Time Considerations

Spectrophotometric measurements of CD and CT values in oils or lipid extracts requires ~45 min depending on the number of samples to be analyzed. The classical PV determination requires a bit of time for preparation of reagents and standardization of the sodium thiosulfate solution. The analysis itself is not very time consuming (~30 min), but in all, about 3 to 3.5 hr should be anticipated. The FOX method has an assay time of <10 min; however, with sample preparation and time to construct the calibration curve, ~2 to 3 hr should be expected.

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Chromatographic Analysis of Secondary Lipid Oxidation Products

One of the factors responsible for the quality deterioration of foods is lipid oxidation. All foods containing fat are susceptible to oxidative rancidity, which can render the food unacceptable to the senses and also reduce nutritional quality.

The extent of oxidative deterioration will determine the acceptability of a food product. Because of this, methods for determining the degree of oxidation are very useful to the food industry. There are many possible methods that can be utilized (see Commentary); however, due to the stability of some of the end products, and their direct relationship with rancidity, headspace GC provides a fast and reliable method for oxidation measurement. Headspace techniques include static, dynamic, and solid-phase microextraction (SPME) methods.

The protocols described in this unit can be conducted to evaluate the volatile lipid oxidation products present in the headspace of raw or cooked meat samples. Results obtained utilizing this methodology can serve as indicators of the oxidation of meat products. Hexanal, which is formed from the oxidation of omega-6 (ω-6) unsaturated fatty acids (linoleate C18:2), is often found to be a major compound in the volatile profile of meat products and is often chosen as an indicator of oxidation in meats, especially during the early oxidative changes (Shahidi, 1994); however, with foods high in ω-9 or ω-3 fatty acids, nonanal or propanal, respectively, may be more appropriate. When choosing a headspace technique, the level of sensitivity required will determine the method. Due to the constant purging involved with dynamic techniques (see Basic Protocol 2), it is possible to achieve higher sensitivity (lower detection limits) as compared with static headspace measurements (see Basic Protocol 1). SPME offers an additional advantage in that the volatile compounds can be concentrated prior to GC analysis, which will also enhance sensitivity (see Alternate Protocol). The heating time and temperature protocols selected for these techniques are based on the characteristics of the sample and the overall objectives of the research. Specifically, the three protocols described here have different levels of sensitivity and will therefore have varied heating parameters. For example, the more sensitive methods will require less volatiles to be produced (less extensive heating) while the less sensitive methods will require more volatiles to be produced (more extensive heating).

VOLATILE LIPID ANALYSIS UTILIZING STATIC HEADSPACE GAS CHROMATOGRAPHY

BASIC PROTOCOL 1

Static headspace GC involves heating the sample in an air-tight environment until the volatile lipids in the food reach an equilibrium with those in the surrounding air. The air above the sample (headspace) is then sampled and analyzed. Flame ionization detection (GC-FID) can be used for quantification and mass-selective detection (GC-MS) can be used for compound identification. This protocol also outlines semiquantitative and quantitative approaches for determination of volatile lipid concentration, and is particularly designed for analysis of a meat sample.

Materials

Meat sample
0.1 µg/µl pure standard in hexadecane for use as an internal standard (standardized semiquantitative analysis)

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0.1 µg/µl pure external standard (e.g., hexanal, pentanal, or 2,3-octanedione) in hexadecane for use as a reference standard (standardized quantitative analysis)
Reproducibility sample (unoxidized food sample; optional)

5-mm grinder
6-ml headspace vials
Teflon/Butyl-lined rubber septa
Crimp seals (Fisher)
Crimping device (Fisher)
Perkin Elmer HS-6 headspace sampler or equivalent
30-m × 0.32-mm i.d. × 1.0-µm film thickness DB-5 GC capillary column or equivalent
Helium
GC equipped with a split injector, flame ionization detector or mass selective detector, and suitable chromatography data station

1. If working with frozen samples, remove samples from the freezer and defrost at room temperature. Trim sample of all subcutaneous fat and connective tissue and grind twice using a 5-mm grinder.
2. Weigh 2 g of sample into a 6-ml headspace vial.
3. If desired, select and add 1 to 10 µl (depending on the sample) of 0.1 µg/µl pure internal standard (IS) in hexadecane directly to the sample.

   Suggested internal standards include n-decane (Ajuyah et al., 1993), 2-heptanone (Jensen at al., 1998), 4-methyl 1-pentanol (Stapelfeldt et al., 1993) and 4-heptanone (Fritsch and Gale, 1977). Selection of the standard is performed such that there will be no coelution with any of the volatiles present within the sample, which will vary depending on species. Hexadecane is utilized as a solvent based on the fact that it will not coelute with the volatiles of interest.

4. Using crimp seals and a crimping device, seal the vial with a Teflon/Butyl-lined rubber septum with the Teflon side facing the sample.
5. Heat at 100°C for 30 min in a Perkin-Elmer HS-6 headspace sampler (or equivalent) and pressurize for 4 min.
6. Inject volatile lipids for a period of 1 min onto a 30-m × 0.32-mm i.d. × 1.0-µm film thickness DB-5 capillary gas chromatography column using helium as the carrier gas. Program the oven to hold the temperature at −20°C for 1 min and then ramp the temperature to 220°C at a rate of 6°C/min. Set the injector and the detector temperatures at 260°C and 280°C, respectively. Use a split ratio of 5:1 with a column head pressure of 15 psi.
7. Collect the data and integrate the detector signal using a suitable chromatography data station.

   Compounds can be tentatively identified by performing the analysis on a GC equipped with a mass selective (MS) detector. Identification can be confirmed by verifying retention times with pure standards.

8a. *For nonstandardized semiquantitative analysis:* For direct comparison between treatments, compare peak areas as a relative indicator of volatile lipid concentration to assess the effect of sample treatment.

8b. *For standardized semiquantitative analysis:* For semiquantitative analysis of known compounds of interest (positively identified in the sample), compare the volatile lipid

data of the internal standard (IS) and the unknown as a ratio of concentration versus peak area:

$$\frac{\text{concentration of IS}}{\text{peak area of IS}} = \frac{\text{concentration of unknown}}{\text{peak area of unknown}}$$

For those compounds that are positively identified and for which pure standards are available, improved accuracy can be obtained by calculating response factors relative to the IS (AOCS, 1974). Response factors for those compounds in the sample to be quantified can be determined as follows: 0.5 μg/μl injections (done in triplicate) of 0.1 μg/μl preparations of the known volatiles in hexadecane are made. Response factors are calculated by comparing peak areas of the known quantities of known volatiles to the peak area of the internal standard per the following formula:

$$\text{Response factor} = \frac{\text{conc. of known volatile standard}}{\text{peak area of known volatile standard}} \times \frac{\text{peak area of IS}}{\text{conc. of IS}}$$

Once determined, the concentration (as determined above) is multiplied by the response factor. This quantification technique is used when the primary objective is to evaluate treatment effects on lipid oxidation, and greater accuracy is desired.

8c. *For standardized quantitative analysis:* For the determination of the actual concentration of the compounds of interest (positively identified in the sample), utilize spiked samples:

i. Select an unoxidized sample of the food to be analyzed as a reproducibility sample. Analyze a minimum of 5 times as described in steps 1 to 7 to establish "baseline" peak areas for volatiles of interest.

ii. Determine a background level for the compounds of interest by averaging the "baseline" peak areas for these compounds known to be present initially in the reproducibility sample.

iii. Dilute standards of the compounds to be quantitated and the internal standard (selected as described in step 8b) to 1 μg/μl in hexadecane, and add (spike) into the reproducibility sample.

iv. Analyze the spiked reproducibility sample a minimum of 5 times as described in steps 1 to 7. Calculate the response factor, as described in step 8b, relative to the internal standard, and adjust for matrix interaction.

Equilibrium of headspace volatile lipids is influenced by a number of factors including the solubility of the component in the food matrix and the vapor pressure over the food system. This relationship may be very complex and must be determined experimentally by spiking the sample with known quantities of the compound of interest and subtracting background levels (Reineccius, 1993).

BASIC PROTOCOL 2

VOLATILE LIPID ANALYSIS UTILIZING DYNAMIC HEADSPACE GAS CHROMATOGRAPHY

Dynamic headspace GC utilizes a constant purge of the sample with an ultra-high purity gas (i.e., helium). The purged volatiles are then adsorbed onto a "trap," followed by heat desorption onto the GC for analysis. Either a flame ionization detector or mass selective detector can be used. The protocol presented here is designed to analyze a meat sample.

Materials

100°C heating block
Tekmar LSC-2000 purge-and-trap concentrator packed with Tenax, or equivalent, with appropriate sampling tube
30-m × 0.32-µm i.d. × 1.0-µm film thickness fused-silica capillary column
GC equipped with a split injector, flame ionization detector or mass selective detector, and suitable chromatography data station

Additional reagents and equipment for headspace gas chromatography (see Basic Protocol 1)

1. Prepare sample (see Basic Protocol 1, step 1). Weigh 2 g of sample into a 6-ml headspace vial and seal with a Teflon/Butyl rubber septum, with the Teflon side facing the sample.
2. Heat the sample at 100°C for 30 min in a heating block in order to produce thermal oxidation products.
3. Transfer the contents of the vial into a sampling tube, add 10 ml of water, and stir briefly.
4. Attach the sampling tube to a purge-and-trap concentrator (dynamic headspace).
5. Heat the sample for 30 min at 30°C while purging helium at a rate of 25 ml/min. Collect volatile compounds on the trap (packed with Tenax or equivalent) and thermally desorb at 180°C onto a 30-m × 0.32-µm i.d. × 1-µm film thickness fused-silica capillary column. After desorption is complete, hold the initial temperature for 1 min at −20°C and then program the temperature to ramp to 220°C at 6°C/min. Set the injector and the detector temperatures at 260°C and 280°C, respectively. Use helium as carrier gas at a flow rate of 3.0 ml/min and a split ratio of 20:1.
6. Collect data and identify using 0.1 µg/µl pure standards as described (see Basic Protocol 1, step 7 and 8).

ALTERNATE PROTOCOL

VOLATILE LIPID ANALYSIS USING SPME GAS CHROMATOGRAPHY

The main principle behind headspace solid-phase microextraction (HS-SPME) is the partitioning of analytes between the sample matrix, headspace, and fiber coating. The chemical potential difference of the analytes among these three phases creates the driving force that moves the analytes from their matrix to the fiber coating. SPME sampling involves extraction of compounds from the food matrix onto a chemically modified fused silica fiber. The compounds are then thermally desorbed in the inlet of the gas chromatograph. The SMPE technique for headspace volatiles as outlined by Gruen et al. (1998) is discussed in this protocol.

Materials *(also see Basic Protocol 1)*

3 M KCl
35°C water bath
SPME fiber (100 µm polydimethylsiloxane coating)

1. Prepare sample (see Basic Protocol 1, step 1). Weigh 1 g of sample into a 6-ml headspace vial.
2. Add 1 ml 3 M KCl and seal the vial with a Teflon/Butyl rubber septum with the Teflon facing the sample.
3. Incubate in a 35°C water bath for 30 min.
4. Insert the SPME fiber into the headspace for 10 min.
5. Thermally desorb in the injector of a GC by placing the needle through the injector septum and then exposing the SPME fiber to the heated inlet.
6. Set-up GC as described (see Basic Protocol 1, step 6).
7. Collect data and identify compounds as described (see Basic Protocol 1, step 7 and 8).

COMMENTARY

Background Information

Oxidation results from the interactions between atmospheric oxygen and the double bonds of unsaturated fatty acids. Several parameters can catalyze lipid oxidation, while others can prevent or slow down the reactions. Metals, light, moisture and heat can all enhance oxidation, while antioxidant compounds (e.g., BHT and vitamin E) can be utilized to retard oxidation. Oxidation of double bonds leads to intermediate peroxides that eventually break down into a variety of stable compounds.

The mechanisms behind lipid oxidation of foods has been the subject of many research projects. One reaction in particular, autoxidation, is consistently believed to be the major source of lipid oxidation in foods (Fennema, 1993). Autoxidation involves self-catalytic reactions with molecular oxygen in which free radicals are formed from unsaturated fatty acids (initiation), followed by reaction with oxygen to form peroxy radicals (propagation), and terminated by reactions with other unsaturated molecules to form hydroperoxides (termination; O'Connor and O'Brien, 1994). Additionally, enzymes inherent in the food system can contribute to lipid oxidization.

The major cause of deterioration of food products is lipid oxidation, from which low-molecular-weight, off-flavor compounds are formed. This deterioration is often caused by the oxidation of the unsaturated lipids present in foods. Off-flavor compounds are created when the hydroperoxides, formed during the initial oxidation, are degraded into secondary reaction compounds. Free radicals are also formed which can participate in reactions with secondary products and with proteins. Interactions with the latter can result in carbonyl amino browning (Lillard, 1987). Lipid oxidation affects many characteristics of foods besides flavor, including color, texture, and nutritive values (Shahidi, 1994).

Rancidity measurements are taken by determining the concentration of either the intermediate compounds, or the more stable end products. Peroxide values (PV), thiobarbituric acid (TBA) test, fatty acid analysis, GC volatile analysis, active oxygen method (AOM), and sensory analysis are just some of the methods currently used for this purpose. Peroxide values and TBA tests are two very common rancidity tests; however, the actual point of rancidity is discretionary. Determinations based on intermediate compounds (PV) are limited because the same value can represent two different points on the rancidity curve, thus making interpretations difficult. For example, a low PV can represent a sample just starting to become rancid, as well as a sample that has developed an extreme rancid characteristic. The TBA test has similar limitations, in that TBA values are typically quadratic with increasing oxidation. Due to the stability of some of the end-products, headspace GC is a fast and reliable method for oxidation measurement. Headspace techniques include static, dynamic and solid-phase microextraction (SPME) methods. Hexanal, which is the end-product formed from the oxidation of Ω-6 unsaturated fatty acids (linoleate), is often found to be a major compound in the volatile profile of food products, and is often chosen as an indicator of oxidation in meats, especially during the early oxidative changes (Shahidi, 1994).

An advantage of headspace GC is that, through an evaluation of the volatiles produced, information regarding the specific fatty acid or

Table D2.2.1 Troubleshooting Guide for Headspace Chromatography of Lipid Oxidation Products

Problem	Possible cause	Solution
Flat baseline, no signal	FID not lit, or flame put out by excessive moisture injected onto column	Relight detector
Tailing peaks	Column degradation	Evaluate column using standards
Poor reproducibility	Inadequate pressurization/equilibration	Ensure samples are heated and pressurized for the same amount of time
		Make sure crimp seal is on tightly
		Check sample homogeneity
Carry-over between injections	Contaminated needle	Clean or replace needle assembly
	Contaminated transfer line	Clean transfer line and heat
	Contaminated septum or inlet	Replace septum, clean inlet

acids present in the food product that is being oxidized is provided. For example, Selke et al. (1975a) heated tristearin in air at 192°C, and showed that methyl ketones and aldehydes were the predominant compounds formed, above their threshold concentrations, along with minor concentrations of *n*-alkanes, primary alcohols, gamma lactones, and monobasic acids. The aldehydes, alkanes, and alcohols were proposed to have been generated from decomposition of monohydroperoxides and coordination with other radicals, while methyl ketones were proposed to have originated from the beta-oxidation of the fatty acid chain followed by decarboxylation.

A study using triolein under the same conditions produced seven major volatile compounds from oleate (heptane, octane, heptanal, octanal, nonanal, 2-decenal, and 2-undecenal) as well as other minor components. Addition of stearate to the reaction substrate produced added volatiles previously identified as originating from tristearin alone (Selke et al., 1977).

Conducting the same experiment using trilinolein produced volatiles unique to the trilinolein substrate, with the major classes being alkanals, 2-alkenals, 2,4-alkadienals, and hydrocarbons. Those volatiles, produced uniquely from this substrate and attributable to the breakdown of 9- and 13-hydroperoxides, include pentane, pentanal, 1-pentanol, hexanal, 2-hexenal, 3-hexenal, 2-heptenal, 2-octenal, 2,4-decadienal, and acrolein. Addition of triolein afforded the added production of volatiles previously identified in triolein alone, but addition of tristearin produced no further tristearin-specific compounds (Selke et al., 1980).

Critical Parameters

Due to the volatility of some of the compounds present in food, it is very important to utilize cryogenic cooling when the sample is introduced onto the GC column. This helps to prevent the loss of low-molecular weight volatiles and also tends to focus volatiles on the initial portion of the column, thus allowing for improved separation and quantification. The use of a film thickness of 1.0 mm will also aid in the retention of the aforementioned compounds. In the static headspace procedure, the 4-min pressurization step is also crucial, in that equal pressures between the sample vials and the GC must be attained to ensure reproducible sample injections. For both the static and SPME procedures, heating the samples for 30 min prior to injection is important to ensure proper equilibration between the sample and the headspace.

Troubleshooting

Table D2.2.1 lists a number of problems that might arise in headspace chromatography of lipid oxidation products, along with their possible causes and solutions.

Anticipated Results

Depending on the source, age, and condition of the food, the volatile profile will vary substantially. There are, however, certain com-

Table D2.2.2 Summary of Volatile Profiles from Various Meat Products

Reference	Meat source	Procedure	Measure	Total volatiles	Hexanal
Thongwong et al. (1999)	Beef: cooked top round	Dynamic headspace: Dynatherm	Peak area[a] $\times 10^5$	420.6 at day 0 928.5 at day 2	24.9 at day 0 326.4 at day 2
Jensen et al. (1988)	Pork: cooked loin	Static headspace: HP7694 HS Sampler	mg/kg DM[b]		0.88: control; 0.91: rapeseed + vitamin E in animal diet
Ahn et al. (1999)	Turkey: cooked thigh	Dynamic headspace: Tekmar 3000	Peak area[a] pA × sec	687.9 at 40°C purge temp; 3233.5 at 80°C purge temp	213.9 at 40°C purge temp; 1460.9 at 80°C purge temp
Turner and Larick (1996)	Chicken: sous vide breast	Static headspace: Perkin Elmer HS-6	Peak area[a] mV/sec $\times 10^6$	3.52 at day 0 4.52 at day 28	
Larick et al. (1992)	Pork: cooked loin	Static headspace: Perkin Elmer HS-6	Peak area[a] mV/sec $\times 10^5$	11.55 with 6.10% linoleic acid in animal diet; 7.81 with 1.76% linoleic acid in animal diet	4.76 with 6.10% linoleic acid in animal diet; 3.16 with 1.76% linoleic acid in animal diet
Ajuyah et al. (1993)	Chicken: cooked breast	Vacuum distillation; and Sep-Pak trapping	ng/g[c]	363 at day 0 2156 at day 15	64 at day 0 314 at day 15
Butler and Larick (1993)	Low-fat beef gels	Static headspace: Perkin Elmer HS-6	Peak area[a] mV/sec $\times 10^5$	18.9 in control gels; 8.3 in gels with nitrite and rosemary oleoresin	6.1 9 in control gels; 2.4 in gels with nitrite and rosemary oleoresin

[a]Peak area equals area under the peak for all peaks combined (total) or for the hexanal peak only.

[b]mg/kg DM equals milligrams of total volatiles or hexanal per kilogram of sample an a dry matter basis.

[c]ng/g equals nanograms of total volatiles or hexanal per gram of sample.

pounds that will almost always be present. Table D2.2.2 is a summary of results taken from several research articles and represents a variety of different meat products.

Headspace volatiles from meat products can be generally grouped into 6 major classes: aldehydes, hydrocarbons, ketones, alcohols, sulfides and branched hydrocarbons (BHC). Aldehydes and hydrocarbons, including BHC, are the most represented classes in pork meat as reported by numerous authors (Mottram, 1985; Yasuhara and Shibamoto, 1990). Ketones and alcohols are the result of the thermal degradation of the hydroperoxides and primary oxidation products of fatty acids (Frankel, 1984; Frankel and Gardner, 1989). Branched hydrocarbons as well as unsaturated and saturated straight-chain hydrocarbons, ranging from C6 to C12, are also found in meat products (Larick et al., 1992). Oxidation studies revealed that saturated hydrocarbons arise through thermal decomposition and, even in small amounts (0.08 ppm), short-chain hydrocarbons were related to rancidity (Warner et al., 1974). In regard to the aldehydes, straight-chain and branched alkanals and alkenals, ranging from C4 to C10, may also be detected; pentanal, hexanal, and heptanal are representative products. Larick et al. (1992) reported that the linoleic acid content in the diet especially influenced the amount of these aldehydes. They were, in fact, related to the autoxidation-decomposition of this fatty acid that also is one of the main acids in soybean oil. Drumm and Spanier (1991) reported the presence of aromatic compounds in beef which seem to be the result of the thermal degradation of amino acids. Moreover, straight-chain methyl ketones (C4, C5, C6, C7, and C8) and a branched methyl ketone (6-methyl-2-heptanone) might also be detected, as well as several straight-chain alcohols (C4, C5, C6, and C8). These compounds are also generated by thermal degradation of hydroperoxides (Selke et al., 1975b). Ketones have been reported to be responsible for off-flavors in meat (Drumm and Spanier, 1991). Sulfur-containing compounds, dimethyl disulfide and dimethyl trisulfide, may also be present. Sulfides have been identified in beef as constituents of cooked ground patties

(Spanier and Drumm Boylston, 1994) and they are responsible for a cabbage-like aroma.

Time Considerations

The time required for heating (to establish equilibrium in the vial) or for purging (to strip volatiles from the sample) is 30 min followed by 5 min of pressurization and injection. The actual GC analysis requires 40 min. Samples can be analyzed such that one sample can be heated or purged while another sample is analyzed by GC, provided each sample is injected after the same amount of heating/pressurization each time.

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This author reported that individual aldehydes may be good indicators of oxidation in food products with hexanal being utilized for foods high in ω-6 unsaturated fatty acids, while for foods high in ω-9 or ω-3 fatty acids, nonanal or propanal, respectively, may be more appropriate.

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Assessment of Oxidative Stability for Lipids

The term oxidative stability refers to the susceptibility of a food or edible oil to lipid oxidation, which causes rancid odors and flavors. Thus, the oil stability index (OSI; Basic Protocol) is an attempt to predict the length of time before a sample will go rancid. The test may be used to provide information regarding the efficacy of antioxidants, the effect of impurities, and evaluation of refining processes of fats and oils. In general, a common approach to determining oxidative stability involves holding samples under accelerated conditions (often elevated temperature) of storage, and measuring lipid oxidation products over a period of time. The storage time that elapses before a rapid increase in lipid oxidation occurs is termed the induction period. The measurement of lipid oxidation during accelerated storage may be performed using tests such as peroxide value (*UNIT D2.1*), hexanal in the sample headspace, and sensory analysis. In contrast, the OSI measures lipid oxidation by monitoring the conductivity of water in which lipid volatiles are trapped.

OIL STABILITY INDEX

BASIC PROTOCOL

A sample is heated, usually at or above 100°C, while air is bubbled through it, and the volatiles created are transferred to a water trap where conductivity is measured (Fig. D2.3.1). The induction period endpoint is determined by the time it takes for the sample to begin a rapid increase in conductivity. The time required for the sample to reach its induction period endpoint is termed the Oil Stability Index (OSI). The Oil Stability Index is Official Method Cd 12b-92 of the American Oil Chemists' Society (AOCS, 1996).

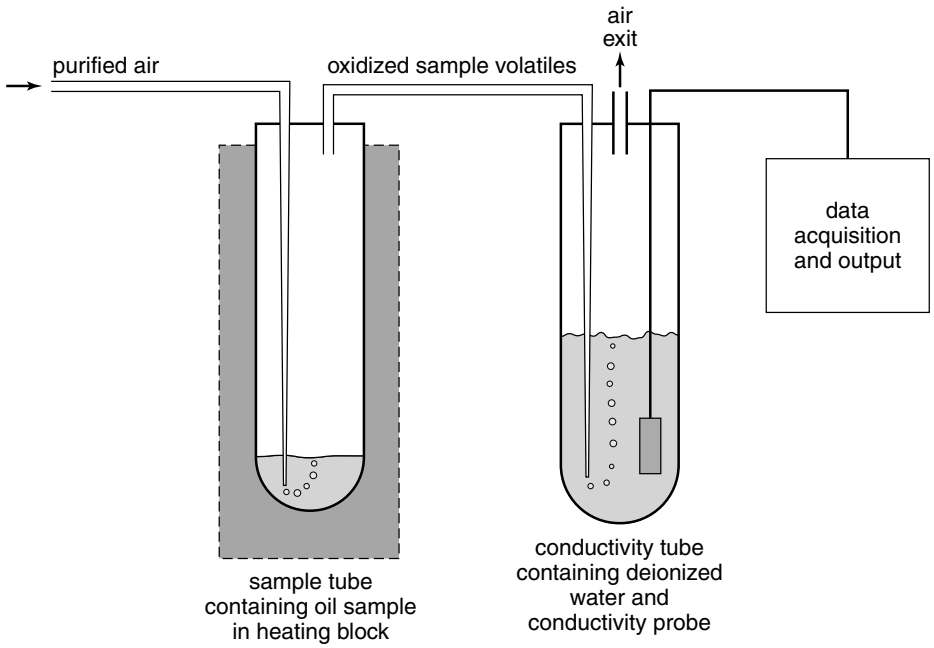

Figure D2.3.1 Schematic diagram of oil stability index (OSI) instrumentation.

Contributed by Oscar A. Pike

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Materials

Detergent that leaves no contaminating residue on glassware (e.g., RBS from Pierce, or Micro from International Products)
Deionized water with conductivity <5 μS-cm^{-1} (1 S = 1/Ω)
Fat sample for calibration
Fat-containing sample of interest (~10 g)

Rancimat (Brinkmann; holds 3 or 6 sample tubes) *or* Oxidative Stability Instrument (Omnion; available from Archer Daniels Midland; holds up to 24 sample tubes)
25 × 200–mm test tubes, with 2-hole stoppers and aeration tubes
Nylon test tube brush
Bubble or digital gas flow meter
Stainless steel container for cleaning tubes
Compressed air source, ~10 psi, with water and hydrocarbon traps (e.g., Model 75-45 Purge Gas Dryer for FTIR and Model A963-000 Trace Hydrocarbon Remover, both available from Balston)

NOTE: This method relies on either of two commercially available instruments that consist of a means of controlling sample temperature (maintained at ±0.1°C), an air distribution system, conductivity tubes, stoppers and probes, and electronics or software for measuring conductivity and determining induction period. The following steps are generally applicable to both the Rancimat and the Oxidative Stability Instrument; however, some variations exist due to differences in hardware and models available. Consult the manufacturer's instructions for additional detail.

1. Position the instrument in a well ventilated room or exhaust hood and connect compressed air source with water and hydrocarbon traps to the air distribution system of the instrument.

   Good ventilation or a hood is needed to removed rancid odors.

2. Clean conductivity tubes and materials. If the equipment will not be used for some time, store dry and covered, and rinse with distilled water before use.

   Omnion recommends that polycarbonate conductivity tubes, conductivity probes, glass aeration tubes, and silicone tubing be soaked in very hot soapy tap water (1% detergent) for ~1 hr, rinsed in hot tap water, soaked in hot tap water for an additional 1 hr, and finally rinsed with distilled water and allowed to soak in distilled water until used.

3. Check the conductivity of deionized water (conductivity <5 μS-cm^{-1}) in a cleaned tube 30 min after placing the water in the tube. If it exceeds 25 μS-cm^{-1} within 30 min of contact with the tube, reclean.

4. Clean sample tubes using very hot (almost boiling) 1% detergent solution to rinse out tubes, while brushing with a nylon brush, then boil 30 min in 1% detergent solution.

5. Brush tubes again, rinse in hot detergent solution, and rinse in tap water.

6. Rinse in deionized water and allow to soak at least 1 hr in deionized water. Perform a final rinse in fresh deionized water and store dry and dust-free.

   Disposable glassware may also be cleaned as a precaution against contamination. An alternate cleaning procedure based on saponification of fat adhering to the glassware is described in AOCS Method Cd 12b-92.

Calibrate sample temperature and airflow

7. To prepare fat sample for calibration, melt the calibration sample (if it is not liquid) by heating slightly (<10°C) above its melting point. If the food sample consists of large pieces, grind it coarsely.
8. Place 5 g (±0.2 g) liquid or particulate sample into bottom of sample tube, avoiding contact with the sides of the tube.
9. Position a thermometer in the sample, ensuring it does not touch the sample tube. Place sample tube in heating unit.
10. Connect silicone tubing from air distribution system to sample tube.
11. Begin the flow of air through the sample, which should be 2.5 ± 0.1 ml/sec for each sample. Measure air flow using a bubble or digital flow meter, and adjust if necessary.
12. Set instrument thermostat and turn on heating unit. Allow for temperature equilibration, then take a reading from the thermometer. Maintain sample to within ±0.1°C of the test temperature, which is commonly 100° to 130°C. Adjust thermostat if necessary.

    The sample temperature must be determined through preliminary experiments. Ideally, it should provide an induction period of between 4 and 15 hr. However, to allow comparison between samples, it may be preferable to have longer induction periods at the commonly used temperatures of 110° or 130°C.

    At least 15 min is needed to ensure temperature equilibration after the unit reaches test temperature.

    Discard this sample used for calibration of temperature and airflow.

13. Prepare conductivity tubes by placing 50 ml deionized water into each tube, then positioning the conductivity probe through the stopper and into the water.

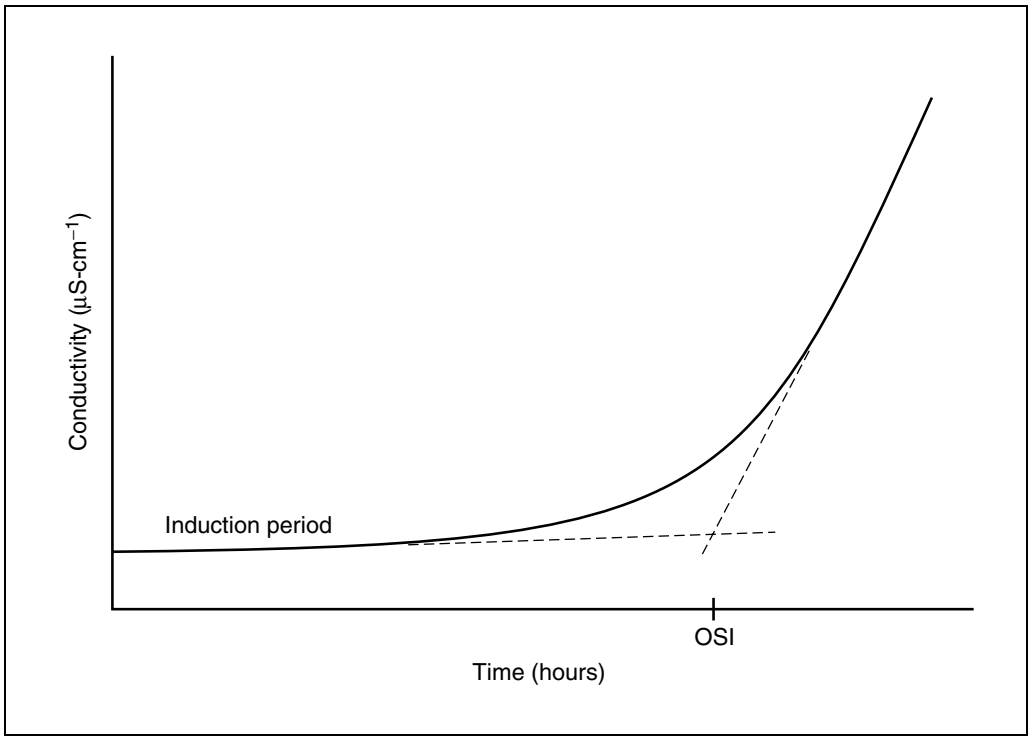

Figure D2.3.2 Data from conductivity probe and determination of oil stability index (OSI) by drawing tangents.

Perform analyses

14. Prepare unknown samples and sample tubes. If a fat sample is not liquid, melt the sample by heating slightly (<10°C) above its melting point. If a food sample consists of large pieces, grind it coarsely. Place 5.0 ± 0.2 g of liquid or particulate sample into the bottom of the sample tube. Avoid sample contact with the sides of the tube.

15. Start the determination. Place sample tubes into the heating block and connect the tubing from the air distribution system to the sample tubes, and from the sample tubes to the conductivity tubes. Position the aeration tubes ~5 mm from the bottom of the sample tubes and the conductivity tubes.

16. Gather the conductivity data for each probe continuously via computer or chart recorder, creating a plot of conductivity over time.

    The Oil Stability Index (OSI) is defined as the time required to reach the induction period's endpoint, which is the point of maximum change in conductivity. The computer software determines this point mathematically by finding the maximum of the second derivative of conductivity plotted against time. It can be approximated by graphing the data, then drawing tangents to the baseline and the sloped line of data representing the rapid increase in oxidation (Fig. D2.3.2). The intersection of the tangents is an approximation of the induction period endpoint, and thus the OSI.

COMMENTARY

Background Information

The oil stability index (OSI) method provides results in a matter of hours instead of months (required for studies done at ambient temperatures). These OSI results are useful as comparative measures of oxidative stability, i.e., to determine the effect of a treatment or antioxidant compared to a control sample. Meaningful predictions of the actual shelf lives of specific commodities require that such shelf life studies be performed at ambient conditions. If only accelerated tests are to be performed, two or more tests based on different principles of lipid oxidation measurement should be conducted; the effect of accelerated storage temperature should also be investigated.

This method has been criticized because of the high temperatures typically used to accelerate the determination (Frankel, 1998). First, the rapid increase in conductivity is due to the volatiles, primarily formic acid, produced in the heated samples. However, formic acid is not formed during lipid oxidation at ambient conditions. Second, antioxidants can be destroyed or volatilized at the high temperatures typically used. Finally, lipid peroxides are not stable, especially at elevated temperatures.

The OSI replaced the Active Oxygen method (AOM), which is no longer recommended by the American Oil Chemists' Society. The AOM required frequent measurement of peroxide value. Not only was this laborious, but determining peroxide value resulted in a discontinuous measurement of lipid oxidation and thus an inexact endpoint to the induction period (arbitrarily set to be the time when a given peroxide value, e.g., 50, was reached). Because of familiarity and common use, product specifications will often list the AOM value, which can be correlated with OSI values. OSI values obtained at 110°C are 40% to 45% of the AOM values obtained at 97.8°C (Stauffer, 1996).

The OSI was primarily developed for oil or fat samples. It is possible to use the method on lipids extracted from foodstuffs; however, such lipids may not have the same oxidative stability as the food since the physical structures are different. Additionally, muscle foods oxidize rapidly at elevated temperature due to physical and chemical alterations during cooking; thus, this method probably will not be useful with raw meats.

Limited work using this method has been performed with some low-moisture foodstuffs such as corn chips, potato chips, crackers, margarine, and butter. Samples containing moisture are problematic inasmuch as water distills over due to the accelerated storage temperatures. Emulsified products, such as butter, margarine and mayonnaise, can be tested after demulsification and removal of the water. The separated oil can then be dried using anhydrous magnesium sulfate (AOCS, 1996). Omnion suggests placing a water trap between the sam-

ple tube and the conductivity tube for samples containing moisture.

Other accelerated methods for measuring lipid oxidation include the oxygen bomb test and the Schaal oven test. These methods are described by Wan (1995).

Critical Parameters and Troubleshooting

It is essential that glassware not be contaminated with trace metals or other pro-oxidants. If this is a problem, consider disposing of the glass sample tubes after a single use.

If sample tube stoppers become loose during a run (a possible problem caused by the pressure of sample volatility), they can be secured using electrical tape placed around the neck of the sample tube and over the stopper top, between the tubes inserted in the stopper.

For highly stable samples, the long analysis time will result in significant water loss in the conductivity tube. Ensure that the deionized water in the conductivity tube covers the conductivity probe by visually checking the height of the water periodically and adding deionized water as needed (AOCS, 1996).

Omnion indicates the possibility of the conductivity probes losing sensitivity over time. Polishing the metal tips with a fine abrasive, e.g., 600 grit silicon carbide, will restore probe sensitivity.

Anticipated Results

OSI results range from 4 hr at 110°C for refined, bleached, deodorized oil, to 80 hr at 110°C for high-stability frying oil (Stauffer, 1996). Because the OSI can be determined at various temperatures, the temperature of the test should always be specified when reporting OSI results.

The coefficient of variation for data from a single instrument should be <5% and for data from several laboratories, ~10% (AOCS, 1996).

Time Considerations

The cleaning procedures will take at ~2 to 3 hr, depending on the number of samples being prepared. Using disposable sample tubes saves time and effort. An additional 30 min is needed to ensure the cleanliness of the conductivity tubes (step 3). Allow the heating unit to warm up during this preparation period. At least 15 min is needed to ensure temperature equilibration after the unit reaches the target temperature. Sample preparation and weighing ranges from a few minutes for liquid oil samples to ~30 min for melting solid fat samples. Finally, the test itself should take between 4 and 15 hr. Highly stable samples can take 80 hr or longer at 110°C and may be run at a higher temperature, e.g., 140°C, to shorten the time.

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Spectrophotometric Measurement of Secondary Lipid Oxidation Products

Lipids are important macromolecules in food from a nutritional point of view and because they contribute toward the products' flavor, texture, general palatability, and storage stability. Oxidation is a major cause of food deterioration and can result in food lipids becoming rancid. Although microbial and enzymatic deterioration can be controlled to a great extent by storing foods at a low temperature, this storage does not prevent lipid oxidation. Therefore, controlling and monitoring lipid oxidation during processing and holding of finished products is increasingly important due to greater demands for precooked convenience-food items for home, fast-food, and institutional uses.

As outlined in *UNIT D2.1*, lipids are susceptible to oxidation. Consequently, analytical protocols are required to assess their quality. Autoxidation is one of the chief processes by which lipids degrade. During the later stages of this free radical–mediated process, hydroperoxides break down into secondary products; these scission products are generally odoriferous by nature. The changes can be monitored by simple spectrophotometric techniques or by more sophisticated instrumental analysis (e.g., *UNIT D2.2*). This unit limits itself to the spectrophotometric techniques available to monitor the secondary products of lipid oxidation. The 2-thiobarbituric acid (TBA) test has been performed (a) directly on a food product followed by extraction of the colored complexes, (b) on a portion of the steam distillate of a food sample, (c) on aqueous or acid extractions of food samples, and (d) on lipids extracted from food samples. Two molecules of TBA react with one molecule of malonaldehyde (also called malondialdehyde or MA) to produce a red pigment with a maximum absorbance at 532 nm. In Basic Protocol 1, 2-thiobarbituric acid reactive substances (TBARS) are determined by an acid extraction methodology. The assay involves treating a food sample with a trichloroacetic acid/*o*-phosphoric acid solution and then filtering the slurry. An aliquot of the filtered sample is reacted with an excess of TBA and the absorbance of the resultant chromophore is measured spectrophotometrically. Alternate Protocol 1 involves the determination of TBARS by the classical distillation technique. This method, which first gained popularity in the 1960s, involves collecting an aqueous distillate of the food product and then reacting it with excess TBA. Originally the TBA was prepared in glacial acetic acid, but now it is commonly prepared as an aqueous solution. In Alternate Protocol 2, TBARS are determined directly in oils or lipid extracts by the TBA colorimetric assay; however, the test is performed with 1-butanol as the solvent. For a discussion of the choice of TBA assay, see Commentary. As TBARS values increase, this is an indication that oxidation is proceeding.

The results from the TBA test, known in older literature as the TBA number, are usually expressed as mg malonaldehyde/kg sample for methods a to c cited above (note that results have also been reported as μmol malonaldehyge/kg or g sample) and as mg of malonaldehyde per unit of lipid for method d. Since it is known that malonaldehyde is not the only aldehyde present in the sample extract and because other aldehydes are capable of producing the same red pigment with TBA when the conditions are favorable, the TBA number/value is more appropriately expressed as the TBARS value, i.e., mg malonaldehyde equivalents/kg sample. To confuse the matter, the AOCS method, which is based on the protocol reported by Pokorny and Dieffenbacher (1989) and permits the direct determination of TBA value in oils and fats without preliminary isolation of secondary oxidation products, defines the TBA value as the increase of absorbance measured at 532 nm due to the reaction of the equivalent of 1 mg of sample per 1 ml volume with

Contributed by Ronald B. Pegg
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2-thibarbituric acid. Alternatively, some researchers report absorbance values from the assay directly to avoid having to construct a standard curve and to perform recovery experiments.

In Basic Protocol 2, carbonyl compounds formed in oils during oxidation are reacted with 2,4-dinitrophenylhydrazine (DNPH) to yield hydrazone derivatives. The addition of alkanolic base produces a very intense wine-red color. This is due to the quinoidal ion, which can be measured spectrophotometrically. As all types of carbonyl compounds react with DNPH, results of the assay are expressed as micromoles of saturated and unsaturated carbonyl present per gram sample.

BASIC PROTOCOL 1

DETERMINATION OF TBA REACTIVE SUBSTANCES IN FOOD SAMPLES: EXTRACTION METHOD

This method determines the TBARS for a food sample by an extraction methodology. Malonaldehyde and other TBARS are extracted from a food sample using a trichloroacetic acid/*o*-phosphoric acid solution. An aliquot of the filtered extract is reacted with excess TBA. Based on a standard curve, results are expressed as mg malonaldehyde eq/kg sample.

Materials

Food sample, homogenized
Antioxidant solution (see recipe)
TCA reagent (see recipe), ice cold
1:1 (v/v) TCA reagent/water
0.02 M 2-thiobarbituric acid (TBA; Sigma), prepared fresh
0.2 mM TMP (see recipe) in water

Laboratory blender (e.g., Stomacher Circulator; Seward) with sample bags or polyethylene pouches (≥250 ml)
100-ml volumetric flasks
Whatman no. 1 filter paper (≥12.5 cm in diameter)
50-ml polypropylene conical centrifuge tubes
Boiling water bath
Spectrophotometer
Glass cuvettes, 1.00 ± 0.01–cm path length

NOTE: As TBA does not readily dissolve in water, gentle heating of the solution may be necessary when preparing 0.02 M TBA. Alternatively, sonicating the flask in an ultrasonic water bath works extremely well for getting TBA into solution. TBA is sometimes referred to as 4,6-dihydroxy-2-mercaptopyrimidine.

Prepare extract

1. Accurately weigh (and record the weight of) 5 g homogenized food sample into a polyethylene pouch or sample bag for a laboratory blender.

   Each sample should be assayed at least in duplicate.

   Filtra-bags (e.g., VWR Scientific Products, Fisher Scientific) contain a highly condensed filter that divides the pouch into two equal compartments. The sample can be inserted into one compartment of the bag and the extract removed from the second. Depending upon the sample in question, this prefiltration step may help speed up gravity filtration through Whatman no. 1 filter paper (step 5).

2. Add 2.5 ml antioxidant solution and 50 ml ice-cold TCA reagent to the pouch and mix the sample 2 min using the laboratory blender.

Generally, duplicate samples are mixed at the same time to speed up the process. The bottles containing the TCA reagent and distilled water are placed in plastic buckets containing ice, and a 50-ml Brinkmann dispensette is connected to each.

Alternatively, a homogenizer can be used if a laboratory blender is not available.

3. Add 50 ml ice-cold distilled water and mix the slurry with the blender for another minute.
4. Place a glass or plastic funnel over the top of a 100-ml volumetric flask and line the funnel with Whatman no. 1 filter paper. Prewet the filter paper with 1:1 TCA reagent/water.
5. Pour the contents from the pouch into the funnel and filter the extract.
6. Fill the flask to mark with 1:1 TCA reagent/water. Mix solution thoroughly.

Perform TBA reaction

7. Pipet a 5.0-ml aliquot from the 100-ml volumetric flask into a 50-ml polypropylene conical centrifuge tube. Add 5.0 ml of 0.02 M TBA. Prepare a reagent blank (i.e., 5.0 ml each of 1:1 TCA reagent/water and 0.02 M TBA) at the same time.
8. Cap the tubes, vortex, and heat 35 min in a boiling water bath.
9. Cool under running tap water for 10 min or by immersion in ice water for ~5 min.

Measure sample absorbance

10. Turn on a spectrophotometer and set the wavelength to 532 nm. Allow instrument to warm up ≥30 min before taking any readings.
11. Zero spectrophotometer with the reagent blank and then measure the absorbance of the test sample using a glass cuvette.

Prepare standard curve

12. Transfer a series of aliquots (0.5 to 5 ml) of 0.2 mM TMP to separate 100-ml volumetric flasks, and fill each to mark with 1:1 TCA reagent/water.

    TMP, sometimes referred to as malonaldehyde bis(dimethyl acetal), is an acetal of malonaldehyde. In the presence of an acid solution, this chemical is hydrolyzed, and malonaldehyde is liberated. Consequently, TMP or its ethyl analog, 1,1,3,3-tetraethoxypropane, can be used in the construction of a TBARS standard curve.

    The 0.2 mM TMP solution is at a concentration of 0.2 mM malonaldehyde. A typical standard series should consist of five samples, ranging from 1×10^{-6} to 1×10^{-5} M malonaldehyde.

    A sample containing 0 ml TMP (i.e., 5 ml each of 1:1 TCA reagent/water and TBA) should be prepared as a reagent blank for zeroing the spectrophotometer.

13. Perform TBA reaction (steps 7 to 9).
14. Measure absorbance of each standard at 532 nm and plot against moles malonaldehyde in the reaction (5-ml aliquot added in step 7). Determine the slope of this curve.

Determine malonaldehyde recovery

15. Repeat steps 1 to 6 with three new homogenized food samples, adding 1.5, 3.0, and 4.5 ml of 0.2 mM TMP, respectively, before step 2. In step 3, reduce the volume of ice-cold distilled water added to each pouch accordingly.

    Recovery of malonaldehyde (equivalents) is determined by spiking samples with known volumes of 0.2 mM TMP.

16. Add 1.5, 3.0, and 4.5 ml of 0.2 mM TMP to three separate 100-ml volumetric flasks and fill to mark with 1:1 TCA reagent/water.

17. Perform TBA reaction and measure absorbance (steps 7 to 11) using the three TMP-spiked samples (step 15) and the three TMP dilutions (step 16).

18. Use the absorbance of the original (unspiked) food sample (step 11) to correct for the endogenous malonaldehyde content and then compare the spiked values to those in the TMP dilutions. The percent recovery is calculated as follows:

$$\% \text{ recovery} = 100 \times A_{SP}/A_{TMP}$$

where A_{SP} is the absorbance of the spiked food sample (corrected for endogenous malonaldehyde) and A_{TMP} is the absorbance of the corresponding TMP dilution.

The three resulting percent recoveries are then averaged and used in step 19.

Determine TBA value

19. Calculate K, a constant derived from the assay, using the following equation:

$$K = [(\text{mol MA}/5 \text{ ml})/A_{532} \times \text{MA mol. wt.} \times \text{DF} \times 10^6 \times (100/\% \text{ recovery})]/m$$

where (mol MA/5 ml)/A_{532} is represented by 1/slope of the standard curve (step 14), the mol. wt. of malonaldehyde is 72.03 g/mol, DF is the dilution factor, 10^6 converts the units so that results can be expressed as mg malonaldehyde eq/kg sample, % recovery is the average value determined in step 18, and m is the sample mass (in grams).

For this example, the equation can be written as: $K = [(1/\text{slope}) \times 72.03 \text{ g/mol} \times (100 \text{ ml}/5 \text{ ml}) \times 10^6 \times (100/\% \text{ recovery})]/5 \text{ g} = 2.88 \times 10^{10}/(\text{slope} \times \% \text{ recovery})$.

20. Calculate the TBA value using the following equation, which expresses the result as mg malonaldehyde eq/kg food sample:

$$\text{TBA value} = K \times A_{532}$$

where A_{532} is the absorbance of the test sample at 532 nm.

ALTERNATE PROTOCOL 1

DETERMINATION OF TBA REACTIVE SUBSTANCES IN FOOD SAMPLES: DISTILLATION METHOD

This method determines the TBARS for a food sample. A steam distillate of an acidified food sample is obtained and an aliquot is then reacted with excess TBA. Based on a standard curve, results are expressed as mg malonaldehyde eq/kg sample.

Additional Materials *(also see Basic Protocol 1)*

0.1 and 4 N HCl
Antifoam A emulsion (e.g., Sigma)

500-ml round-bottom flasks
Glass beads
Standard distillation column with condenser
Heating mantel

1. Accurately weigh (and record the weight of) 10 g homogenized food sample on weighing paper and transfer the sample and paper to a 500-ml round-bottom flask.

Each sample should be assayed at least in duplicate.

2. Add to the flask:

   95 ml distilled water
   2.5 ml antioxidant solution
   2.5 ml of 4 N HCl (final 0.1 N, to adjust pH to ~1)
   a few drops of antifoam A emulsion
   3 to 5 glass beads (to prevent bumping).

   Swirl contents well.

3. Attach a standard distillation column with a condenser and distill rapidly using a heating mantle until 50 ml distillate is collected.

   The distillation step generally takes 15 to 20 min.

4. Perform TBA reaction on 5 ml distillate and measure absorbance (see Basic Protocol 1, steps 7 to 11), but prepare the reagent blank in water instead of TCA reagent/water, and increase heating time to 45 min.

   If the A_{532} of the sample is >1, then a smaller sample mass or a more dilute stock solution should be used and the test repeated.

5. Prepare a standard curve, measure malonaldehyde recovery, and calculate TBA value as described (see Basic Protocol 1, steps 12 to 20), using the modifications indicated above.

   Adjusting for changes in dilution factor and sample mass, $K = [(1/slope) \times 72.03\ g/mol \times (50\ ml/5\ ml) \times 10^6 \times (100/\%\ recovery)]/10\ g = 7.2 \times 10^9/(slope \times \%\ recovery)$.

DETERMINATION OF TBA REACTIVE SUBSTANCES IN OILS OR LIPID EXTRACTS: DIRECT METHOD

ALTERNATE PROTOCOL 2

This method permits the direct determination of TBARS in oils and fats without preliminary isolation of secondary oxidation products. This protocol is applicable to animal and vegetable fats and oils, fatty acids and their esters, partial glycol esters, and similar materials (AOCS, 1998).

CAUTION: As prolonged exposure of 1-butanol can lead to headaches and faintness, it is recommended that as much of the analysis as possible, particularly sample preparation, be carried out in a fume hood.

Additional Materials *(also see Basic Protocol 1)*

Oil or lipid extract sample (*UNIT D1.1*)
1-Butanol (containing <0.5% water), ACS spectrophotometric grade
0.2% (w/v) 2-thiobarbituric acid (TBA) in 1-butanol, sonicated to dissolve TBA and stored up to 1 week at 4°C
0.2 mM TMP (see recipe) in 1-butanol

25-ml volumetric flasks
Screw-cap glass test tubes
95°C water bath

Prepare sample

1. Accurately weigh (and record the weight of) 50 to 200 mg oil or lipid extract sample into a 25-ml volumetric flask. Dissolve the sample in a small amount of 1-butanol and bring to volume with 1-butanol. Mix solution thoroughly.

   If an ultrasonic water bath is available, sonicate flask to ensure uniform mixing.

Perform TBA reaction

2. Transfer 5.0 ml sample solution to a dry screw-cap glass test tube. Add 5.0 ml of 0.2% TBA in 1-butanol and cap the tube. Prepare a reagent blank (i.e., 5.0 ml each of 1-butanol and 0.2% TBA) at the same time.

   Samples should be prepared in duplicate.

3. Vortex and incubate 2 hr in a 95°C water bath.

4. Remove tubes from the bath and cool under running tap water for 10 min (until they reach room temperature).

Measure sample absorbance

5. Turn on a spectrophotometer and set the wavelength to 532 nm. Allow the instrument to warm up ≥30 min before taking any readings.

6. Zero spectrophotometer with the reagent blank and then measure the absorbance of the test sample using a glass cuvette.

   If the A_{532} of the sample is >1, then a smaller sample mass or more dilute stock solution should be used, and the test repeated.

Prepare standard curve

7. Transfer a series of aliquots (0.1 to 1 ml) of 0.2 mM TMP prepared in 1-butanol to separate 25-ml volumetric flasks, and fill each to the mark with 1-butanol.

   TMP, sometimes referred to as malonaldehyde bis(dimethyl acetal), is an acetal of malonaldehyde. In the presence of an acid solution, this chemical is hydrolyzed and malonaldehyde is liberated. Consequently, TMP or its ethyl analog, 1,1,3,3-tetraethoxypropane, can be used in the construction of a TBARS standard curve.

   The 0.2 mM TMP solution is at a concentration of 0.2 mM malonaldehyde. A typical standard series should consist of five samples, ranging from 1×10^{-6} to 1×10^{-5} M malonaldehyde.

   A sample containing 0 ml TMP (i.e., 5 ml each of butanol and TBA) should be prepared as a reagent blank for zeroing the spectrophotometer.

8. Perform TBA reaction (steps 2 to 4).

9. Measure absorbance of each standard at 532 nm and plot against moles malonaldehyde in the reaction (5-ml aliquot added in step 2). Determine the slope of this curve.

Determine TBA value

10. Calculate K, a constant derived from the assay, using the following equation:

    $K = [(\text{mol malonaldehyde/5 ml})/A_{532} \times \text{malonaldehyde mol. wt.} \times \text{DF} \times 10^6]/m$

    where (mol malonaldehyde/5 ml)A_{532} is represented by 1/slope of the standard curve (step 9), the mol. wt. of malonaldehyde is 72.03 g/mol, DF is the dilution factor, 10^6 converts the units so that results can be expressed as mg malonaldehyde eq/g sample, and m is the sample mass (in mg).

    For this example, the equation can be written as:
    $K = (1/\text{slope}) \times 72.03\ \text{g/mol} \times (25\ \text{ml}/5\ \text{ml}) \times 10^6]/m = (3.60 \times 10^8)/(\text{slope} \times m)$.

11. Calculate the TBA value using the following equation, which expresses the result as mg malonaldehyde eq/g oil:

    $\text{TBA value} = K \times A_{532}$

    where A_{532} is the absorbance of the test sample at 532 nm.

12. Alternatively, calculate the TBA value according to the AOCS method, which expresses the result as the increase of absorbance measured at 532 nm due to the reaction of the equivalent 1 mg of sample per 1 ml volume with TBA.

    TBA value = $(50 \times A_{532})/m$

    where the factor 50 is based on the volume from a 25-ml volumetric flask and a cuvette path length of 1 cm, A_{532} is the absorbance of the test solution (already corrected based on reagent blank), and m is the mass (in milligrams) of the test sample (AOCS, 1998).

DETERMINATION OF CARBONYL COMPOUNDS IN FOOD LIPIDS

BASIC PROTOCOL 2

This method describes the quantitative determination of carbonyl compounds in fats and oils. It is based upon the formation of 2,4-dinitrophenylhydrazones of carbonyl compounds in the presence of a trichloroacetic acid (TCA) catalyst, followed by colorimetric determination of the hydrazone compounds (i.e., as quinoidal ions) in an alkanolic basic solution.

Materials

Oil or lipid extract sample (*UNIT D1.1*)
Benzene (carbonyl-free)
4.3% (w/v) trichloroacetic acid (TCA) in benzene, stored up to two weeks at room temperature
0.05% (w/v) 2,4-dinitrophenylhydrazine (DNPH) in benzene, stored up to several months at 4°C
KOH solution (see recipe)
Absolute ethanol (carbonyl-free)

50-ml volumetric flasks
60°C water bath
Spectrophotometer
Glass cuvettes, 1.00 ± 0.01–cm path length

CAUTION: Benzene has been listed as a carcinogen by the Environmental Protection Agency. Avoid contact with skin and work in a fume hood to avoid inhalation of benzene vapors.

NOTE: Solutions and extracts of oil or lipid samples should be protected from undue exposure to light and air before use in order to prevent deterioration of the existing carbonyl compounds as well as further oxidation of the lipid.

1. Accurately weigh (and record the weight of) 50 to 80 mg oil or lipid extract sample in a 50-ml volumetric flask. Dissolve oil sample in 5.0 ml benzene.

   Each sample should be assayed at least in duplicate.

   Edible oils are easily dissolved in benzene, but if a solid food is to be analyzed, the sample should be well ground first. A portion is accurately weighed, to which a known volume of benzene is added. The sample is mixed, centrifuged, and a 5.0-ml aliquot of this extract is used for the analysis.

2. Pipet 3.0 ml of 4.3% TCA in benzene into the 50-ml volumetric flask followed by 5.0 ml of 0.05% DNPH in benzene. Prepare a reagent blank (i.e., 5.0 ml each of benzene, 4.3% TCA in benzene, and 0.05% DNPH in benzene) at the same time.

3. Stopper the flask, heat 30 min in a 60°C water bath, and then cool to room temperature.

   This solution is stable for several hours.

4. Turn on a spectrophotometer and set the wavelengths to 430 and 460 nm. Allow the instrument to warm up ≥30 min before taking any readings.

5. To develop the color, add 10 ml KOH solution and dilute to volume with carbonyl-free absolute ethanol. Mix solution thoroughly and let stand for 10 min.

   Stagger the preparation of duplicate samples so that readings will be taken after exactly 10 min.

6. While the sample is incubating, zero the spectrophotometer with the reagent blank. After exactly 10 min, read the absorbance of the test sample at 430 and 460 nm using a glass cuvette.

7. Calculate the results as μmol carbonyl/g sample based on the following equations:

$$C_U = (3.861 \times A_{460} - 3.012 \times A_{430})/0.854$$

$$C_S = 3.861 \times A_{460} - 2.170 \times C_U$$

$$C_T = C_U + C_S$$

   where A_{460} and A_{430} are the sample absorbances at 460 and 430 nm, respectively, and C_U, C_S, and C_T are the unsaturated, saturated, and total carbonyl contents, respectively.

   In order to express the absorbances obtained by this method in terms of moles of carbonyl, the spectral absorbances of DNPH derivatives of several purified carbonyl compounds were measured. At 430 nm, molar extinction coefficients of 16,000 and 21,350 were determined for the saturated and unsaturated carbonyl compounds, respectively, whereas at 460 nm, the values were 12,450 and 28,100, respectively. Based on the present methodology and use of 1-cm cuvettes, the above equations were derived (Henick et al., 1954).

   The concentration of dissolved lipid should not exceed 2.5×10^{-4} M carbonyl. The assay should be repeated if the value is too high.

REAGENTS AND SOLUTIONS

Use ACS-grade chemicals unless otherwise specified and deionized or distilled water in all recipes and protocol steps. Basic suppliers for chemicals would include VWR Scientific Products, Fisher Scientific, and Sigma. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antioxidant solution

Weigh 0.5 g propyl gallate (0.5% final) and 0.5 g ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA; 0.5% final) into a 100-ml volumetric flask, dissolve crystals in a small volume of 1:1 (v/v) ethanol/water and dilute to mark with ethanol/water solution. Prepare fresh daily.

KOH solution

Dissolve 4 g KOH (4% final) in 100 ml carbonyl-free ethanol with the aid of gentle heating and stirring. Filter solution through fine glass wool with suction. Prepare fresh daily.

Alternatively, sonicating the flask in an ultrasonic water bath works extremely well to get the KOH into solution.

1,1,3,3-Tetramethoxypropane (TMP), 0.2 mM

Prepare a 20 mM TMP stock solution by accurately weighing 328 mg TMP (Sigma) into a 100-ml volumetric flask and diluting to mark with water for Basic Protocol 1 and Alternate Protocol 1 or 1-butanol for Alternate Protocol 2 (20 mM malonaldehyde final). Store up to 3 months at 4°C. Prepare a 0.2 mM working solution by diluting 100-fold in a volumetric flask. Store up to 1 month at 4°C.

continued

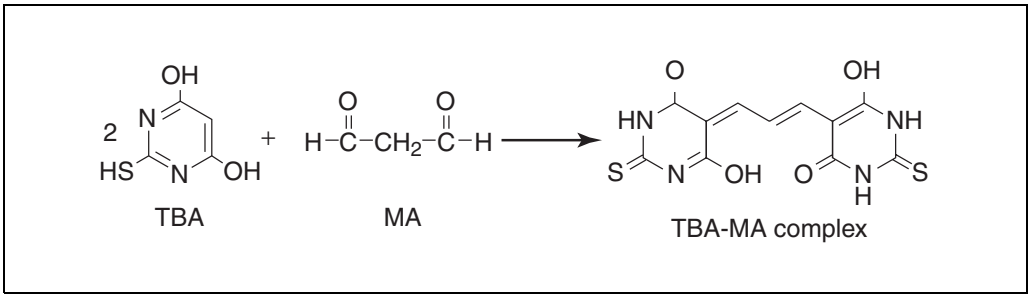

Figure D2.4.1 Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA) in the classical TBA test.

TMP is also referred to as malonaldehyde bis(dimethyl acetal). 1,1,3,3-Tetraethoxypropane, or malonaldehyde bis(diethyl acetal), can be substituted for TMP. Stock and working solutions are prepared as for TMP.

Trichloroacetic acid (TCA) reagent

Weigh 200 g TCA (20% final) in a beaker and dissolve crystals in a small volume of water. To a 1-liter volumetric flask, add some water and then add 16 ml 85% (w/w) *o*-phosphoric acid (1.6% final). Swirl the solution and then quantitatively transfer the TCA mixture from the beaker to the volumetric flask using water. Fill to mark with water and mix contents thoroughly. Store up to ~4 to 6 weeks at 4°C.

CAUTION: *As well as being hygroscopic, TCA crystals are dangerous. Care should be exercised.*

Addition of perchloric acid to the TCA solution is believed to assist in releasing TBA reactive substances from meat.

COMMENTARY

Background Information

Lipids are susceptible to oxidation and, as such, require analytical protocols to measure their quality. As described in *UNIT D2.1*, autoxidation is one of the chief processes by which lipids degrade. The primary products from this reaction are hydroperoxides. These odorless and colorless transient species break down by various means to secondary products, which are generally odoriferous by nature. Being able to measure secondary oxidation products by simple spectrophotometric means is important for the food scientist so that he or she is able to characterize the extent of lipid oxidation. However, the researcher should be cautioned that one assay (e.g., TBA test) does not provide all the answers. To get a better picture of the story, both primary and secondary products of lipid oxidation should be assessed simultaneously by the different methods available (*UNIT D2.1*).

TBA assay

A classical assay for the semiquantitative estimation of oxidation of edible lipids is the 2-thiobarbituric acid (TBA) test. This spectrophotometric determination was first described by Kohn and Liversedge (1944) in a paper on the oxidation products of animal tissues and then reported in detail in a paper on the oxidation of meat lipids by Tarladgis et al. (1960). The assay involves the isolation of malonaldehyde, a relatively minor but important 3-carbon dialdehyde ($C_3H_4O_2$) in oxidized foods, and its subsequent reaction with TBA, which forms a pink adduct with a distinctive absorption maximum at 532 nm (Figure D2.4.1).

Results are commonly expressed as the TBA value or number, that is, mg malonaldehyde eq/kg sample; however, there are some problems associated with this approach. Calculated TBA values should not be interpreted as absolute levels of rancidity. This stems from an older paper in the literature that indicates that a TBA value >1 is an indication of rancidity. As will be described below, a number of nonoxidation substances can interfere with the TBA assay and give falsely high readings. TBA values will also vary as a function of the method employed (e.g., extraction versus distillation). For this reason, researchers should prepare their own standard curves, carry out recovery experiments, and calculate TBA values on a concen-

tration basis. An alternate approach has been to report absorbance data or the percent inhibition of lipid oxidation relative to control samples. Even though the TBA test has some drawbacks, as discussed below, this now classical assay is one of the most frequently employed methods for determining lipid oxidation in foods because of its simplicity and relative speed.

Various protocols and modifications have been reported in the literature on how to perform the TBA test. In foodstuffs, malonaldehyde can be bound to various constituents of the food (e.g., proteins), and therefore it must somehow be released prior to determination. It is difficult to determine the optimal conditions for release of malonaldehyde as they differ from one material to another and require different conditions for hydrolysis. Heat and/or strong acid are thought to be essential for the liberation of malonaldehyde from precursors or bound forms, for condensation with TBA, and for maximal color development. For edible oil samples or lipid extracts, the test is simplified in that samples are directly dissolved in butanol and then an aliquot is reacted with TBA. Alternatively, a food sample can be heated with TBA solution and the red pigment that is formed can be extracted from the reaction mixture with butanol or a butanol/pyridine solution (Turner et al., 1954; Sinnhuber and Yu, 1958; Placer et al., 1966; Uchiyama and Mihara, 1978; Ohkawa et al., 1979; Pokorny and Dieffenbacher, 1989).

Three basic approaches have been employed to conduct the TBA test on foods. It can be performed (1) on aqueous acid extracts of food samples (see Basic Protocol 1; Tarladgis et al., 1964; Witte et al., 1970; Siu and Draper, 1978; Caldironi and Bazan, 1982; Salih et al., 1987; Bedinghaus and Ockerman, 1995), (2) on an aliquot of a steam distillate of food samples (see Alternate Protocol 1; Tarladgis et al., 1960; Igene et al., 1979; Yamauchi et al., 1982; Ke et al., 1984), and (3) on the extracted lipid portion of food samples (see Alternate Protocol 2; Younathan and Watts, 1960; Pikul et al., 1983). The chromophore formed by the reaction of malonaldehyde with TBA is then quantified spectrophotometrically.

Commonly, 1,1,3,3-tetramethoxypropane (TMP) or its tetraethoxy analog is used as a standard. Under acidic conditions these acetals are hydrolyzed to 1,3-propanedial (i.e., malonaldehyde) and therefore can be used in the construction of a TBA reactive substances (TBARS) standard curve; one mole of malonaldehyde is released for each mole of TMP.

Extraction, distillation, and direct methods

Of these three basic approaches, the aqueous acid extraction TBA test (see Basic Protocol 1) may be considered the better assay for estimating the malonaldehyde content in food. In this technique, the food sample is not subjected to heat and less sample autoxidation takes place; the malonaldehyde content of the sample should not be overestimated. For this reason, the extraction assay has been listed as the basic protocol to determine TBA values of foodstuffs.

At times, however, interfering substances are extracted into solution. For the assay to work properly, the sample extract must be a clear liquid for spectrophotometric analysis and must contain quantitative amounts of the malonaldehyde-TBA chromophore. Presence of water-soluble proteins, peptides, pigments, amino acids, and fat droplets in the extract necessitates a filtration step that can be tedious and not completely effective. Turbidity of the medium containing the malonaldehyde-TBA adduct will inflate absorbance readings and consequently cause overestimation of TBA values. For such problem samples, the distillation technique (see Alternate Protocol 1) may be the only option available to assess TBA values. In the extraction procedure, only TBARS that are instantaneously released from the food matrix into the acid/aqueous phase are determined. Percentage recovery experiments are necessary. However, having said this, a number of TBA investigations have shown that the extraction method offers a definite advantage over the distillation procedure in that greater recoveries of malonaldehyde are generally attained. Moreover, the extraction assay is faster and easier to perform than the distillation technique and does not require expensive distillation equipment.

The distillation method (see Alternate Protocol 1) involves recovering malonaldehyde from an acidified food product. It is similar to that of the direct heating approach (see below), except that TBA is reacted only with an aliquot of the distillate. Consequently, physical and chemical interference by extraneous food constituents in the reaction with TBA is minimized because the food is never directly in contact with TBA. Unfortunately, direct heating of a food under acidic conditions enhances the degradation of existing lipid hydroperoxides as malonaldehyde precursors, and generates additional reactive radicals and scission products other than malonaldehyde that can react with TBA (Raharjo and Sofos, 1993). More malonaldehyde or reactive substances will be gener-

ated and the TBA values obtained will be inflated and not represent a true assessment of the malonaldehyde content in the food sample. Whether the distillation step is carried out by conductivity heating or by incorporation of steam can significantly impact the TBA values obtained. Addition of a synthetic antioxidant such as propyl gallate, *t*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), or butylated hydroxyanisole (BHA) prior to heating may help prevent further oxidation of the food sample during the assay. Nonetheless, the distillation method reported by Tarladgis et al. (1960) is still commonly used as the standard method for TBA analysis even though it is the most cumbersome (Pikul et al., 1989).

Lipids can be extracted from foods by the Bligh-Dyer method (Bligh and Dyer, 1959) and then an aliquot of the extract reacted with TBA (Alternate Protocol 2). The advantage of this direct procedure, like that of the distillation method, is that the presence of interfering substances such as soluble proteins, peptides, amino acids, and pigments in food samples is eliminated. However, due to the hydrophilic nature of malonaldehyde, only a minor portion of it will be present in the organic lipid extract; bound malonaldehyde may not be extracted. Moreover, removal of the organic solvent necessitates heating, which could result in further lipid oxidation and malonaldehyde formation. Therefore, when the direct method is used for TBA analysis, the majority of malonaldehyde present in the sample is produced by the assay conditions. In other words, data on the level of malonaldehyde present in the original food sample are no longer available (Raharjo and Sofos, 1993). Addition of a synthetic antioxidant prior to heating may help retard further generation of TBARS.

Specificity and significance of TBA assay

Malonaldehyde formation and accumulation in foodstuffs depend on the degree of unsaturation of polyunsaturated fatty acids (PUFA), malonaldehyde precursors from nonlipid origin, types of oxidation catalysts and conditions, and the reactivity of malonaldehyde with other biological materials (Raharjo and Sofos, 1993). Of these factors, the degree of fatty acid unsaturation of the food lipids markedly influences the extent and amount of malonaldehyde that can be generated from oxidized PUFA. With increasing levels of unsaturation, more malonaldehyde will be generated during autoxidation. Consequently, researchers should be cautioned against using TBA values as an indicator of lipid oxidation when comparisons of oxidation are being made between foods with significantly different fatty acid compositions.

The TBA test was once believed to be specific for malonaldehyde (Tarladgis et al., 1960, 1964; Lai et al., 1995), but this is not so. In fact, the TBA method has been criticized as lacking specificity and adequate sensitivity toward malonaldehyde (Raharjo and Sofos, 1993). A variety of aldehydes such as alkanals, alk-2-enal, alk-2,4-dienals, and 4-hydroxyalkenal, as well as dioxolanes and furan derivatives, are generated as scission products of lipid hydroperoxides (Kosugi et al., 1988). Many of these can react with TBA to form a pink chromogenic adduct with an identical absorption maximum as the TBA-malonaldehyde complex (Marcuse and Johansson, 1973; Kosugi and Kikugawa, 1986; Witz et al., 1986; Kosugi et al., 1987, 1988). Baumgartner et al. (1975) reported that when the TBA test was applied to a mixture of acetaldehyde and sucrose a 532 nm–absorbing chromogen was produced. This adduct was indistinguishable from that formed by the condensation of malonaldehyde with TBA.

In certain products a secondary absorption peak occurs around 450 to 460 nm. If the compounds giving rise to this absorbance are of sufficient concentration, the secondary peak may overlap the malonaldehyde-TBA peak causing erroneously high TBA values (Sinnhuber and Yu, 1977). For example, Kosugi and co-workers (Kosugi and Kikugawa, 1986; Kosugi et al., 1987, 1988) found that condensation reactions between 1-hexanal and 1-heptanal and TBA in 15% acetic acid at 100°C yielded not only a red pigment but also an unstable yellow one with an absorption maximum between 450 and 470 nm. The reaction of alk-2-enals such as 2-hexenal and 2-octenal with an excess of TBA under acidic conditions produced yellow, orange, and red chromophores.

The interaction of malonaldehyde with amino acids and the presence of other nonlipid TBA-reactive compounds in biological samples may interfere with the quantitation of oxidation products in the TBA test. Due to uncertainty concerning the exact identity of compounds that can react with TBA, the ambiguous term TBARS is now commonly used in lieu of TBA number or value (Ke et al., 1984; Gray and Pearson, 1987; Guillén-Sans and Guzmán-Chozas, 1998). Because TBA is not specific for malonaldehyde alone, certain limitations exist when performing the test for evaluation of the

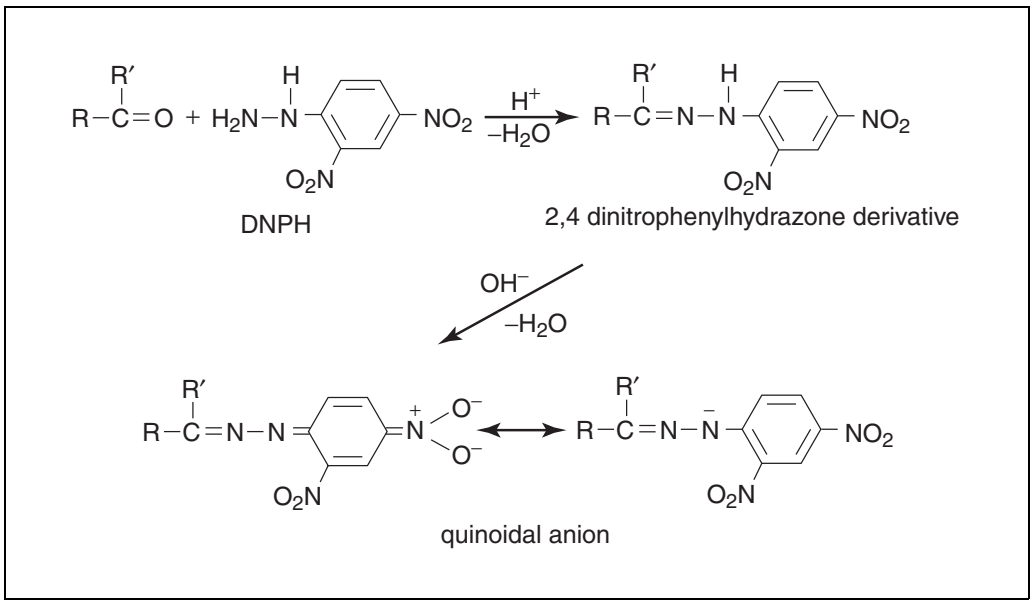

Figure D2.4.2 Development of the quinoidal ion from the reaction of 2,4-dinitrophenylhydrazine (DNPH) with an aldehyde or ketone. Adapted from Meyer and Rebrovic (1995) with permission from AOCS Press.

oxidative state of foods and biological systems due to the chemical complexity of these systems. To compensate for these factors, numerous modifications of the original TBA test have been reported in the literature (Marcuse and Johansson, 1973; Ke and Woyewoda, 1979; Robles-Martinez et al., 1982; Pokorny et al., 1985; Tomás and Funes, 1987; Schmedes and Hølmer, 1989), but this raises a host of new problems.

Sample preparation, types of acidulants and their concentrations in the reaction mixture, pH of the reaction mixture, composition of the TBA reagent, length of the TBA reaction, and possible use of antioxidants and chelators in the systems are among the factors that may influence results reported by researchers from various laboratories. For example, Moerck and Ball (1974) suggested that Tenox II be added to the distillation mixture prior to heating in order to retard further oxidation and subsequent artifact formation during this step, whereas Ke et al. (1977) reported the use of propyl gallate and EDTA during distillation for this purpose. Pikul et al. (1983) concluded that protection is necessary during TBA assays to prevent sample autoxidation and consequent artifactual high analytical results.

Ideally, an acceptable antioxidant should not only prevent autoxidation of samples during an assay but should also not interfere with either the release of malonaldehyde from preformed fatty acid hydroperoxides or the binding of malonaldehyde with TBA to produce the measured chromophore. Pikul et al. (1983) reported that the addition of 75 µg BHT/mg fat before heating in the distillation procedure was sufficient to prevent sample autoxidation. Furthermore, the autoxidation of sample lipids during extraction was prevented by the addition of 0.01% (w/v) BHT to the 1:2 (v/v) chloroform/methanol reagent. Chicken samples analyzed without any BHT addition yielded six times higher malonaldehyde concentrations compared with samples that received BHT during extraction and 75 µg BHT/mg fat during the TBA assay.

As has been described in this unit, the TBA test is often used to measure the oxidative deterioration of food lipids and, in many cases, the results parallel flavor deterioration of foodstuffs. The relationships between TBARS and sensory scores are important if the assay is to have any significant meaning. A number of studies dealing with such correlations have been reported in the literature, but the TBA test itself has never been adequately standardized as a measure of food acceptability. Furthermore, results obtained from the various protocols are known to be both method and operator dependent. A good number of these investigations have shown significant correlations between sensory scores and the chemical test, but there are variations among these reports. The inconsistent data may be due to the use of different category scales, trained or untrained panelists, and different descriptors (Poste et al., 1986; Love, 1988).

Table D2.4.1 Differences in the Conversion Factor (K)[a] Used in the Spectrophotometric 2-Thiobarbituric Acid (TBA) Test Due to Variability in Recovery and Procedures[b]

% Recovery	K	TBA test	Literature reference
52.7	NR	Distillation	Hoyland and Taylor (1989)
NR	8.95	Distillation (18 days storage)	Resurreccion and Reynolds (1990)
57	NR	Aqueous extraction	Siu and Draper (1978)
NR	8.6	Distillation	Moerck and Ball (1974)
59	NR	Distillation	Wang et al. (1997)
61.4	NR	Aqueous extraction	Wang et al. (1997)
62	NR	Aqueous extraction	Newburg and Concon (1980)
65	NR	Distillation	Kwon and Watts (1964)
NR	8.15	Distillation (35 days storage)	Resurreccion and Reynolds (1990)
NR	8.1	Distillation	Shahidi et al. (1987)
68	7.8	Distillation	Tarladgis et al. (1960)
68.6-77.2	6.2	Distillation	Crackel et al. (1988)
70	NR	Aqueous extraction	Siu and Draper (1978)
71	12.58	Aqueous extraction	Bedinghaus and Ockerman (1995)
73.2	NR	Distillation	Kakuda et al. (1981)
83	NR	Distillation	Siu and Draper (1978)
80-85	NR	Distillation	Shamberger et al. (1977)
90.7	6.5	Distillation	Pikul et al. (1989)
93	6.2	Aqueous extraction	Salih et al. (1989)
NR	6.2	Distillation	Lin et al. (1989)
94	NR	Distillation	Siu and Draper (1978)
94	5.5	Aqueous extraction	Pikul et al. (1989)
94	5.2	Aqueous extraction	Witte et al. (1970)
98-100	NR	Aqueous extraction	Csallany et al. (1984)
97-106	NR	Distillation	Ke and Woyewoda (1979)

[a] K is in mg malonaldehyde eq/kg sample.

[b] Adapated from Raharjo and Sofos (1993) with permission from Elsevier Science. NR signifies not reported.

Lai et al. (1995) tried to account for some of the problems in relating sensory scores to TBA values. These authors suggested that there is too much variability in methods used to determine correlation coefficients (e.g., data pooled within groups, across groups, mean values only) and the number of factors involved in the assays. Because strong effects of sample treatments and storage times are involved, correlations between TBARS and sensory scores should be calculated by pooling information within treatments and storage times. Many significant correlations of TBARS and sensory scores that have previously been reported were calculated on the individual observations over groups differing in mean values of TBARS and sensory scores without considering the impact of the mean differences on the magnitude of correlation.

Assay for carbonyl compounds

The intensity of undesirable sensory notes has been positively correlated with the content of carbonyl compounds formed through lipid autoxidation reactions. In general, the carbonyl compounds present have the greatest impact on flavor owing to their low flavor thresholds in comparison with hydrocarbons, substituted furans, and alcohols. Aldehydes are major contributors to the loss of desirable flavor in meats because of their rate of formation during lipid oxidation and low flavor threshold. Thus, an alternative approach for monitoring the extent of lipid oxidation in fats and oils is to measure

Table D2.4.2 Effect of TBARS Determination Method on TBARS Recovered from Selected Fresh Frozen Meat Sources[a]

Meat source	Distillation (D)				Extraction (E)			Ratio (D/E)
	Tarladgis	Rhee	Moerck	Average	Sinnhuber	Wittes	Average	
Beef patties	4.00	3.28	3.43	3.57	1.34	1.14	1.24	2.88
Chicken breast	0.55	0.46	0.49	0.50	0.21	0.27	0.24	2.08
Chicken thigh	0.52	0.21	0.40	0.38	0.27	0.15	0.21	1.81
Ground beef	0.82	0.74	0.89	0.82	0.57	0.36	0.47	1.74
Ground pork	3.81	3.57	4.01	3.80	1.17	1.62	1.40	2.71
Lamb	1.49	1.99	2.18	1.89	0.74	0.58	0.66	2.86
Pork roast	1.80	2.00	2.09	1.96	0.67	0.89	0.78	2.51
Veal	0.82	0.57	0.65	0.68	0.33	0.18	0.26	2.62

[a]Adapted from Wang et al. (1997) with permission from Food and Nutrition Press. Distillation methods were described by Tarladgis et al. (1960), Rhee (1978), and Moerck and Ball (1974). Extraction methods were described by Sinnhuber and Yu (1977) and Witte et al. (1970). Results are expressed as mg malonaldehyde eq/kg meat sample.

the total carbonyl compounds formed from degradation of hydroperoxides.

One simple spectrophotometric method for their determination is an analysis based on the absorbance of the quinoidal ion, a derivative of aldehydes and ketones (see Basic Protocol 2). This ion is formed from the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl moiety of an aldehyde or ketone, followed by the reaction of the resulting hydrazone with alkali as shown in Figure D2.4.2. The method is useful in the range of carbonyl concentration from 10^{-4} to 10^{-6} M. Although variations in methodology for the choice of solvent, wavelength, or workup in which to analyze the quinoidal ion exist in the literature, the method of Henick et al. (1954), described here, is directly applicable to an oil sample. Based on absorbance measurements at two wavelengths and standard carbonyl compounds, results are expressed as micromoles of unsaturated, saturated, or total carbonyl per gram sample. As with the TBA test, the carbonyl content in oils is usually monitored at discrete time intervals during storage and the contents of saturated, unsaturated, and total carbonyls are compared against one another or with different chemical indices. The CV procedure has been criticized because hydroperoxides decompose under the conditions of the test and thus possibly interfere with the quantitative determination of the carbonyls. Several modifications to this procedure have been offered to overcome the problem, but no ideal solution has been found. As a result, the CV method has not been widely employed in recent years, probably because of inaccuracies in the procedure described, lack of correlation with sensory data, and the development of more sensitive and precise ways of measuring specific volatile carbonyls by means of gas-liquid chromatography (White, 1995; also see *UNIT D2.2*).

Critical Parameters and Troubleshooting

Determination of TBARS using the extraction method

Often the *K* factor is taken for granted, but its accurate determination is of critical importance for converting absorbance readings into TBARS values. Because each procedure employed for the TBA test may lead to different levels of malonaldehyde recovered from an identical sample, the calculated TBA values may also be variable (Raharjo and Sofos, 1993). Different types of samples and procedures for malonaldehyde recovery determination will result in different recovery and conversion factors used for TBA number calculations. This is evident from the scientific literature as summarized in Table D2.4.1 (Raharjo and Sofos, 1993); more details pertaining to this are provided below in the discussion of the distillation method. The main advantage that the extraction method has over the distillation technique is that there is less chance for artifact formation; therefore, more realistic results are obtained and are believed to correlate better with sensory data (note, however, that this is not always the case). It is important that the TCA and water solutions used during the extraction step are kept cold to minimize artifact formation. Again, due to the empirical nature of the assay, by observing the described protocol carefully and analyzing at appropriately timed intervals,

most of these influences can be eliminated or held constant. Thus, fairly repeatable results can be obtained. The extraction procedure may be inadequate in cases of colored samples and samples high in fat, that is, >10% (Hoyland and Taylor, 1991).

Determination of TBARS using the distillation method

As stated above, the *K* factor is often taken for granted. Tarladgis et al. (1960) used a *K* factor of 7.8 for the distillation method based on an average recovery of 68% from meat samples (Table D2.4.1). Each researcher should determine *K* for his or her respective TBA analysis. In the scientific literature, the details on how *K* factors are determined are often neglected or, in some cases, the 7.8 value reported by Tarladgis et al. (1960) has been used by other researchers. *K* is dependent upon many factors of the assay such as the exact distillation apparatus being used (i.e., rate and length of heating required to obtain 50 ml distillate), whether the sample is heated by conductivity or steam, type of food sample being analyzed (i.e., matrix effects), mass of sample tested, sample pH, dilution levels employed, and the average recovery of malonaldehyde from spiked samples. Sørensen and Jørgensen (1996) reported that when optimizing the distillation procedure with regard to distillation principle (i.e., heating by thermal conductivity or steam injection) and rate, the reaction yield was more affected by changes in distillation principle than by changes in distillation rate. Simple distillation (i.e., conduction heating) yielded significantly higher TBA values than steam distillation at the same distillation rate. The reproducibility of the distillation procedure was found to be approximately five to ten times poorer than that of the extraction procedure. Instead of reporting TBARS values, some researchers have simply reported arbitrary absorbance data based on a described procedure or reported TBA results as percent inhibition relative to the control sample. In view of the diversity and empirical nature of methods employed, results from one laboratory cannot be compared to another.

Contamination of glassware by soap residue, resulting in turbid test solutions, can interfere with the assay during the color development step. For this reason, the use of disposable polypropylene centrifuge tubes has been recommended. Any variation in the procedure outlined may affect the results. By observing the protocol carefully and analyzing at appropriately timed intervals, variability in the results can be reduced.

Determination of TBARS in oil using the direct method

The method is not applicable to phospholipid concentrates or to samples containing carbohydrates or proteins that could react with either the reagent or TBA reactive substances. For analysis of such samples, the lipid fraction should be isolated by extraction before the TBA determination, or the volatile TBA reactive substances should be collected by steam distillation. The purity of the reagents should be checked. The absorbance of the reagent blank should not exceed 0.1 units. If it does, a new reagent solution should be prepared using 1-butanol and TBA of better purity. The TBA value determination may be affected by hydroperoxides, oxygen, antioxidants, and trace metals. By observing the procedure carefully and using chemicals of high purity, most of these influences can be eliminated or held constant, and fairly repeatable results can be obtained.

Determination of carbonyl compounds

Solutions and extracts of lipid for the test should be protected from undue exposure to light and air before use in order to prevent deterioration of the existing carbonyl compounds as well as further oxidation of the lipid. The solvents must be of high purity to eliminate any interference from existing carbonyls. Fading of color for the test sample occurs after the addition of the alkanolic base solution. For this reason, absorbance readings should be read exactly 10 min after the alkali addition. If the analysis is performed <1 hr after preparation of the sample, data obtained are reliable.

Anticipated Results

As an example of a food product, TBA values of meat can vary greatly, for example, from <0.1 to >10 mg malonaldehyde eq/kg sample depending upon the type of meat in question, whether it is fresh, cooked, or previously frozen, and how long it has been stored. Table D2.4.2 reports TBARS of various fresh frozen meat sources determined by distillation and extraction methods cited in the literature (Wang et al., 1997). In this study, veal and lean ground beef had been frozen for <3 months; chicken thigh and breast meat for 6 months; and ground pork, low-fat ground beef patties, lamb, and pork roast for 24 months, all at −18°C in PVC overwrap packaging. With the exception of chicken thigh, samples analyzed by distilla-

tion methods produced TBARS values that were significantly higher than those of the extraction methods. This probably demonstrates the greater breakdown of hydroperoxides as a result of high temperatures experienced during distillation. Based on the average TBARS values for each method, the ratio of distillation/extraction method is shown.

Salih et al. (1987) reported that TBA numbers of cooked poultry meat obtained using the distillation method were ~2.6-fold higher than those of the extraction method. These results were comparable to those reported by Witte et al. (1970), who noted that the TBA values determined by the distillation method were about twice as large as those determined by the extraction method. Salih et al. (1987) found that the relationship of both distillation and extraction methods correlated well with panel scores, thereby indicating that TBA is a valid objective test used with sensory evaluation for monitoring warmed-over flavor in poultry meat.

It is important to remember that lipid oxidation is a dynamic process and that TBARS values reflect only one point in this process. The concentration of TBARS for meat tends to increase over the storage period, reach a maximum value, and then decline. When an unknown sample is presented for analysis, caution must be exercised as the sample's placement on this curve is unknown.

Time Considerations

The three TBA assays described in this unit are somewhat time consuming, but are simple to perform. Compared to other assays, a good number of samples can be analyzed in a day (e.g., up to 20 in duplicate), so there is a relative speed to the TBA test. Alternatively, gas chromatography (GC) can be used to measure the volatiles of lipid oxidation (e.g., hexanal levels), or high-performance liquid chromatography (HPLC) can be used to measure malonaldehyde content during increasing storage periods. However, these techniques require time for sample preparation and analysis, sophisticated accessories in some cases (e.g., headspace analyzer for GC, either static or dynamic), and a set-up period to determine appropriate instrumental conditions, as well as qualified personnel to run the analytical equipment. It is important to remember that oxidation is a dynamic process, so TBA data reflect just one point in time. For this reason, TBA assays are generally carried out on samples at selected time intervals (e.g., 1, 3, 5, and 7 days for cooked meat samples), and the trends in treated samples are compared to appropriate controls.

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Key References

Henick et al., 1954. See above.

Describes a simple and sensitive spectrophotometric method to estimate the content of total carbonyl compounds in rancid fats and foods by trapping them with 2,4-DNPH; the technique determines total carbonyls, including those that are nonvolatile, decreasing the ability of the assay to correlate well with sensory data. Although gas chromatographic techniques are better suited for determining volatile carbonyl compounds from lipid oxidation, this is still the classical colorimetric assay.

Pokorny and Dieffenbacher, 1989. See above.

Reports a standardized method for direct determination of TBA value in oils, fats, and lipid extracts. Unlike distillation and extraction methods, this technique determines total reactive substances without previous isolation of the volatile fraction. Furthermore, the solvent of choice is organic (1-butanol) rather than aqueous. This protocol is reported as the official (AOCS) method to determine TBA value (direct method) of animal and vegetable fats and oils, fatty acids and their esters, partial glycol esters, and similar materials.

Tarladgis et al., 1960. See above.

Describes a steam distillation method to determine malonaldehyde content in meat products. An aliquot of the distillate obtained is reacted with acidic TBA reagent and the chromogen formed is measured spectrophotometrically. The intensity of the color was believed to be a measure of the malonaldehyde concentration and hence lipid oxidation. The authors investigated a number of factors affecting the distillation of malonaldehyde such as pH of the material to be distilled, time of heating during distillation, and varying the amount of distillate collected. Other parameters examined included the effect of heating time on color development with the TBA reagent, stability of the distillate and of the colored complex, recovery of malonaldehyde in the distillate, and the correlation between sensory scores and TBA number.

Witte et al., 1970. See above.

Described a new extraction method for determining TBA values for meat during storage. Unlike the classical distillation procedure, this method involves an aqueous extraction of the sample with trichloroacetic acid prior to reaction with TBA. It also described a similar way of calculating the K value so that absorbance readings could be expressed as TBA values. This technique is faster and easier to perform than the distillation method and the authors recommended its employment for rapid analyses of large numbers of samples.

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Determination of Solid Fat Content by Nuclear Magnetic Resonance

Food lipids are mixtures largely comprising a range of structurally similar triacylglycerol molecules with differing fatty acid substituents (Lawler and Dimick, 1998). Because these compounds are to some extent mutually soluble, edible fats typically show a melting range rather than the clear melting point that is characteristic of a pure compound. The melting range of many food lipids occurs at the temperatures used in food preparation, storage, and use. Because the ratio of solid fat to liquid oil affects the texture, stability, and mouthfeel of foodstuffs, the melting range is a parameter of crucial interest to many food scientists involved in research and quality control. The ratio of solid to total fat is expressed as the solid fat content (SFC).

The melting profile is frequently further complicated by the presence of a number of types of metastable crystal habits and by the significant supercooling of liquid oils. A consequence of these factors is that it is impossible to accurately predict the solids content of a real food oil using only temperature and composition data; experimental investigations are therefore essential. Fortunately, many of the physical properties of solids and liquids are very different, and several appropriate techniques for measuring fats in both phases have been developed. The most widely used approach is pulsed nuclear magnetic resonance (NMR; Basic Protocol, direct method). The nuclei in solids have different resonance properties from those in liquids, and the two phases can be distinguished by NMR to provide their relative proportions easily and nondestructively. The indirect method, given in the Alternate Protocol, reads only the liquid signal but at two temperatures—when the sample is completely melted and when it is tempered—and uses the ratio to determine the solid fat content.

The adoption of NMR methods as a standard way to measure SFC is largely due to the work of the American Oil Chemists' Society (AOCS). Over the past decades they have conducted a series of interlaboratory collaborative trials to revise and improve methodologies and have published their recommendations as *Official Methods and Recommended Practices of the AOCS* (Firestone, 1998). The methods described here were based on methods Cd 16b-93 and Cd16-81 of this book, and readers are encouraged to consult the original text. The important advantages of NMR for this application are that it can rapidly and nondestructively measure the number of hydrogen nuclei in the solid or liquid phase and thus the ratio of the amounts of each phase.

CAUTION: No one with a pacemaker should approach within 30 cm of an NMR instrument. Higher specification instruments require further caution. If in doubt, consult a doctor and the equipment manufacturer. Keep magnetic storage devices (including credit cards) away from the measurement coil.

DETERMINING SOLID FAT CONTENT BY A DIRECT PARALLEL NMR METHOD

BASIC PROTOCOL

The "direct" methodology separately measures the NMR signals from nuclei in the solid and liquid phases. The solid fat content is calculated from their ratio.

Materials

Fat samples to be tested
Water baths (or similar apparatuses) capable of maintaining temperatures listed in Table D3.1.1

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Current Protocols in Food Analytical Chemistry (2001) D3.1.1-D3.1.8

Table D3.1.1 Tempering Protocols of Stabilizing Versus Nonstabilizing Fats[a]

Nonstabilizing fats	Stabilizing fats
Melt at 100°C	Melt at 100°C
100°C, 15 min	100°C, 15 min
60°C, 5-15 min	60°C, 5-15 min
0°C, 60 ± 2 min	0°C, 90 ± 5 min
Hold 30-35 min at each measurement temperature[b]	26°C, 40 ± 0.5 hr
	0°C, 90 ± 5 min
	Hold 60-65 min at each measurement temperature[b]

[a]Steps listed in sequential order.

[b]Holding step at measurement temperature should be performed in a dry tempering block to prevent contamination of NMR coil with water.

Pulsed nuclear magnetic resonance spectrometer (NMR), >20 MHz, with maximum dead time plus pulse width of 10 µsec (e.g., Bruker Canada Minispec; Oxford Instruments Analytical QP20+)
Oven set to 100° ± 2°C
Paper filter
Glass NMR tubes, ≥150-mm long with 10-mm diameter
Tempering blocks: 80-mm-thick stainless steel blocks with approximately twenty 70-mm-deep, 10.35-mm-diameter holes, with a center-to-center separation of 17 mm (Firestone, 1998)

1. Using the protocols given in Table D3.1.1, set up a series of water baths at the appropriate temperatures for tempering and measuring samples (see Time Considerations). For all but the last stage, set up a support for the NMR tubes in a temperature-controlled water bath. For the final stage, immerse a tempering block ~60 cm into a water bath, being sure to keep the sample holes dry. Allow to reach thermal equilibrium.

   For stabilizing fats, there is a 40-hr tempering step. In this case, the dry tempering blocks do not need to be preequilibrated until ~1 hr before use (see Time Considerations and Table D3.1.1).

   In general, cocoa butter is considered a stabilizing fat and all others nonstabilizing fats. For more detail on this issue, see Critical Parameters and Troubleshooting.

   The measurement temperatures selected depend on the needs and interests of the investigator. Many semi-solid fats show melting between −10° and 60°C, and measurements within this range are therefore the most useful. Melting properties are best calculated from a set of measurements taken over the relevant range at 5°C intervals. To measure a full profile from a single tempering sequence it is necessary to have a preequilibrated tempering block for each temperature under investigation.

   A dry environment is required for the final tempering stage as liquid water on the outside of the NMR tube can be transferred to the coil and bias the measurement (as well as subsequent measurements taken from the contaminated coil). If a dry tempering block is unavailable, it may be possible to use a water bath if rapid and complete drying of the tube can be achieved before significant temperature drift has occurred.

   Tempering blocks can be easily made by drilling holes in a stainless steel block or other suitable conductive metal. As they are frequently brought to temperature in a water bath, it is worthwhile selecting a metal that is not prone to rust.

2. Use the protocols set out in the instrument manual for a pulsed NMR unit to perform the recommended self-tests and internal calibrations.

3. Melt fat samples in an oven at 100° ± 2°C until the fats are clear (~5 min) with no suspended solids. If necessary, pass the fat through a paper filter and then melt again. Mix thoroughly.

4. Transfer fat samples to glass NMR tubes, filling the tubes to a height of 30 to 50 mm. Ensure that no fat adheres to the outside of the tubes.

5. Temper samples following the protocols in Table D3.1.1. Keep tubes capped during tempering, especially with stabilizing fats. Dry tubes thoroughly and quickly before transferring to the dry tempering blocks at measurement temperatures.

   A parallel setup is generally recommended, in which a different NMR tube is used for each measurement temperature (Table D3.1.1). However, when the amount of sample is limited, it may be necessary to use a serial rather than parallel experimental design. In a serial measurement, after measuring a sample at the first (lowest) temperature, it is transferred to the next warmest tempering block, held at the measurement temperature for the appropriate incubation time, and then remeasured. The process is repeated until the entire temperature range has been covered. Note that the solid fat content of a given sample is a function of thermal history, so serial and parallel measurements may give dissimilar results.

   Tempering is essential to answer questions such as "What is the solids content of an oil at a given temperature?" or to measure a complete melting profile, but it is not always required. When measuring the solid content of a sample drawn from a given stage of a process line or comparing products during storage, the sample may be transferred directly to a sample tube and measured.

6. Remove a sample tube from the tempering block and quickly transfer it to the measurement port.

   The aim is to make a measurement as close to the selected temperature as possible, so speed is essential to minimize temperature changes. Several instruments are fitted with a microswitch to begin the recording regimen as soon as a sample tube is in place, and their use is recommended.

   Make certain that the only material in the measurement coil is the sample under investigation. In particular, ensure that the outside of the tube and the inside of the tempering blocks and sample coils remain dry and clean at all times.

   The total number of samples that can be analyzed in a single experiment is limited by the 5-min time range during which samples may be maintained at the measurement temperature (Table D3.1.1); in practice the maximum is ~20 samples. If it is necessary to record more data, an efficient alternative would be to step tubes through the tempering process one at a time or in small batches so that each sample strictly follows the limits set out in Table D3.1.1.

7. Record four pulses (averaged to reduce signal noise) with a pulse repetition time of 2 sec for nonstabilizing fats. For stabilizing fats, use a single 6-sec pulse.

8. Read the solid fat content (SFC).

   Most commercial instruments are supplied with software to make this calculation internally and, if available, they should be used. If not, the solid fat content can be calculated as:

$$\mathrm{SFC} = \frac{(S_{\mathrm{LS}} - S_{\mathrm{L}}) \times F}{S_{\mathrm{L}} + (S_{\mathrm{LS}} - S_{\mathrm{L}}) \times F + D}$$

where S_{LS} is the NMR signal due to both liquid and solid nuclei (recorded 11 μsec after the pulse), S_L is the NMR signal due to liquid nuclei (recorded 70 μsec after the pulse), F is an empirical correction factor (established during calibration) to account for detector dead time, and D is the digital offset factor (also established during calibration).

9. Repeat steps 6 to 8 for all remaining samples.

10. Report data as a percentage with one decimal place, and record the temperature at which the measurements were made (i.e., record a solid fat content of 80.5% at 20°C as N20 = 80.5%). Report the measurement method with the results.

ALTERNATE PROTOCOL

DETERMINING SOLID FAT CONTENT BY AN INDIRECT PARALLEL NMR METHOD

This technique is used to measure the protons in the liquid phase of the sample. It is possible to determine the SFC by comparing the signal of a completely melted sample with the signal of the same material after tempering. To account for the effect of temperature on instrument sensitivity, it is also necessary to measure an oil known to be completely liquid at both temperatures. The indirect measurement is less reproducible than the direct method, but requires a lower specification instrument.

Additional Materials *(also see Basic Protocol)*

Standard liquid oil: good-quality winterized olive oil
Spectrometer capable of recording a signal ~70 μsec after the pulse

Measure melted samples

1. Set up water baths and preequilibrate tempering blocks as described (see Basic Protocol, step 1).

2. Melt fat samples as described (see Basic Protocol, step 3).

3. Transfer samples and standard liquid oil to NMR tubes. Fill the tubes so that the entire sample is contained within the NMR coil.

   A height of ~1 cm is usually suitable. The exact amount of sample is not crucial.

   Olive oil is prone to oxidation, which may lead to the formation of solids over time. Thus, it should be stored in a cool dark environment and replaced every 2 to 3 months.

4. Heat samples and standard oil to 60°C.

   If both samples are not completely liquid at this temperature (see Basic Protocol, step 3), it may be necessary to increase the temperature to 65° or 70°C.

5. Rapidly transfer first the standard and then the experimental sample to the measurement port of the NMR. Pulse as for the direct method (see Basic Protocol, step 7) and measure the signal 70 μsec after the pulse. Record values as $Standard_{60}$ and $Sample_{60}$, respectively.

6. Repeat step 5 for all remaining samples.

Measure tempered samples

7. Temper fat samples as for the direct method (see Basic Protocol, step 5).

8. Remove the first standard tube from the final tempering block and quickly transfer it to the measurement port. Measure the NMR signal as above and record the value as $Standard_T$.

9. Remove sample tube from the tempering block and quickly transfer it to the measurement port. Measure the NMR signal and record the value as $Sample_T$.

10. Repeat steps 8 and 9 for all remaining samples.

Determine solid fat content

11. Calculate the percentage solid fat content as:

$$\%\ \mathrm{SFC} = 100 - \left(\frac{\mathrm{Standard}_{60} \times \mathrm{Sample}_{T}}{\mathrm{Sample}_{60} \times \mathrm{Standard}_{T}} \times 100 \right)$$

This calculation is based on the assumptions that both the standard and the sample are completely liquid at 60°C, and that the standard is completely liquid at the final measurement temperature. Although these assumptions are probably justified in many cases, they may not always be correct, particularly when making measurements at low temperatures. As a general guideline, liquid oils are clear, so the reference should be clear at the measurement temperature, and both the reference and the samples should be clear at 60°C. If the (filtered) samples are at all turbid at 60°C, it may be possible to raise the reference temperature as noted in step 4. If the reference oil is turbid at the measurement temperature, consult the manufacturer of the NMR spectrometer for an alternative reference.

12. Report data as for the direct method (see Basic Protocol, step 10).

COMMENTARY

Background Information

The two NMR methods described here are based on similar principles (Eads and Croasmun, 1988; Eads and Davis, 1994). All nuclei have a characteristic quantized energy state. Under normal conditions there is a distribution among the available quantum states, but in a strong magnetic field certain nuclei will "line up" in the same state. When a low-energy (radio frequency) electromagnetic pulse is applied to the aligned nuclei, some of them will flip to alternate quantized states. These nuclei will eventually realign in the applied magnetic field, but this process takes time. Each realignment generates its own electrical field, and thus the kinetics of the realignment can be monitored. The relaxation curve recorded is characteristic of both the chemical and physical environments of the nuclei, making it a powerful analytical tool. Nuclei in solid phases have limited molecular mobility, so they relax very quickly (~10 μsec) and can be easily distinguished from the more mobile and slower decaying liquid nuclei. This characteristic is exploited in the direct technique (Basic Protocol), where a first measurement (proportional to the sum of the solid and liquid nuclei) is made quickly after the pulse, and a second reading (of only the liquid nuclei) is made at a later time, when the solid component has decayed. The detector is "blinded" for a short time after the application of the electromagnetic pulse (usually for a few microseconds); therefore, some of the signal from solid fat, which is already decaying during this time, is lost before the first measurement at 11 μsec can be made. The calibration protocol (determination of the F factor) on known samples accounts for this inevitable loss.

The indirect method (Alternate Protocol) involves measuring the same sample twice: once at a temperature where the sample is known to be completely liquid and once after tempering, when some of the fat has crystallized. Although only the liquid signal is read, the ratio of the two measurements gives the SFC. In this case, the use of a liquid calibration oil takes into account the variability of instrument sensitivity with temperature.

In an interlaboratory study, the AOCS reports that the direct method is more reproducible; however, a more-limited, single-laboratory study found no significant difference between the two methods (Mills and van der Voort, 1981). On balance, the direct method is therefore slightly preferred.

The methods recommended here all require a considerable investment in equipment. The best method available in the absence of suitable magnetic resonance equipment is dilatometry (Hannewijk et al., 1964). Dilatometry takes advantage of the fact that solid fat is more dense than the corresponding liquid oil; therefore, the change in volume of a fixed amount of oil with temperature can be used as a precise indication of solids content. Dilatometry remains a recommended method of the AOCS (Method Cd 10-57; Firestone, 1998), but has, to a large extent, been supplanted by the NMR-based

Table D3.1.2 Melting Points of Selected Fats[a]

Fat	Melting point (°C)[b]
Beeswax (white)	65 to 70
Beeswax (yellow)	60 to 65
Carnauba wax	26.0 to 34.5
Corn oil	119 to 131
Cottonseed oil	–3 to 1.6
Olive oil	2 (s)
Peanut oil	1.5 (s)
Rapeseed oil	–10
Sesame oil	–6 to –3
Soybean	–16 to –10

[a]All data from Doss (1952).

[b]Melting point is often defined as the lowest temperature at which there are no solids present. *s*, solidification temperature.

methods. Importantly, dilatometry measures an empirical parameter, the solid fat index (SFI), which is only approximately equal to the true solid fat content as determined by NMR. Various formulae are available to convert between SFC and SFI, but none are generally applicable; therefore, this approach should be used with caution. Note also that dilatometry is a more laborious technique to perform than NMR, and requires the use of hazardous chemicals (as cleaning agents).

Both dilatometry and NMR methods are well described and validated by the AOCS. In addition, success with various other techniques has been reported by other sources. The most important alternative is differential scanning calorimetry (DSC). DSC is a measurement of the relative temperature response of two samples exposed to a similar heating/cooling environment (Wunderlich, 1990). If there is a difference in specific heat between the two samples, there will be a difference in cooling and heating rates. The specific heat of a material increases during a melting phase transition because of the latent heat of fusion and decreases during a freezing phase because of crystallization. The peak in heat flux can be used to measure the melting behavior of a fat, and the percentage of solids at any temperature can be calculated as the fraction of the total peak area (O'Brien, 1998).

DSC has the advantage of being fast (results typically obtained within 1 hr) and suitable for any consistency of fat. However, perhaps because of the difficulty in accurately weighing the small (5 to 15 mg) samples required, precision and reproducibility tend to be poor. DSC instruments can be programmed to accurately reproduce the thermal environment the fat will experience during a processing operation and measure its solids content under these conditions. However, it is rarely possible to conduct a full tempering protocol (see Table D3.1.1), as this would tie up a valuable instrument for a several days to run a single sample.

Another interesting alternative is ultrasonic velocimetry. Sound travels more quickly in solids than liquids, and it is possible to measure the solids content of an oil sample by measuring the time needed for an ultrasonic pulse to move through a fixed pathlength of oil as a function of temperature (McClements and Povey, 1987). Comparisons of ultrasonic with NMR methods have shown the former perform at least as well as the latter, and perform better in the case of low levels of solids (McClements and Povey, 1988). However, because this technique has not been widely adopted or received detailed review by the AOCS or other professional organizations, it is not given the status of a recommended method here.

Another method that has been used with some success is infrared spectroscopy (van der Voort et al., 1996); this technique may be worth considering if the equipment is available. Visible or ultraviolet turbidimetry should not be used to estimate the degree of crystallinity (Marangoni, 1996) in liquid oil.

Critical Parameters and Troubleshooting

In selecting the correct tempering protocol from Table D3.1.1, it is necessary to determine whether the fat under investigation is stabilizing or nonstabilizing. In general, a stabilizing fat has a tendency to form metastable polymorphic forms if cooled rapidly. The formation, melting, and recrystallization of the unstable

polymorphs tend to be complex, kinetically limited processes and are therefore poorly reproducible. The tempering steps are designed to provide a uniform cooling protocol and to allow a reasonable time for the system to approach thermodynamic equilibrium. Stabilizing fats contain appreciable quantities of 2-oleo-disaturated glycerides and include, most importantly, cocoa butter as well as some other specialty fats.

Most problems with solid fat content determination arise from inconsistencies in sample preparation. As noted above, the solids content is dependent on the temperature history of the sample, so deviations from the tempering protocols can lead to inaccuracies. The time required to measure a sample is not expected to lead to extensive crystallization or melting, but care should be taken to start the measurement as soon as possible after the oil is removed from the tempering block. Several instruments can be fitted with a thermostatic control for the sample in the coil, and this should be used if it is reliable. It is recommended that the NMR tubes be capped, particularly for the long tempering times required for stabilizing fats.

NMR instruments often require a considerable warm-up time and are frequently left on constantly to ensure consistent performance. They should be situated on a low-vibration surface and kept close to room temperature. Regular calibration is essential for the success of the direct method. It is good practice to keep a day-to-day record of the F factor of the instrument and use sudden changes as an indication of a problem.

Anticipated Results

The solid fat content will, of course, range between 0% and 100%. The melting range may be as narrow as 2° to 3°C and as wide as several tens of degrees centigrade. To a very limited extent, it is possible to estimate the solids content of an unknown fat based on texture or biological source. At <2% solid fat, the sample will appear to be a liquid oil. A solid fat content between 10% and 25% results in samples that are often soft and pliable (plastic). A higher solids content leads to the formation of an increasingly hard and brittle solid product. The melting points of several common food and nonfood oils and fats are shown in Table D3.1.2. Several fats (importantly milk fat) contain a mixture of high- and low-melting fats and may show a stepped rather than smooth melting curve. It should be stressed that the solids content of a mixture of fats is rarely predictable from the properties of the components. Nonetheless, judgments based on these considerations enable an investigator to decide whether the results of an NMR method are reasonable.

Based on an interlaboratory study, the AOCS estimates that the repeatability of measurements should be 1.3% and the between-laboratory variability 3.3% (Firestone, 1998).

Time Considerations

Nonstabilizing fats require a relatively brief tempering process, so the complete experiment can be conducted in a single day. The tempering of stabilizing fats requires some planning, and the following time line is suggested.

Day 1, 3 p.m.: Prepare sample tubes and proceed through the tempering process. It should be possible to have the samples start the 26°C tempering stage by 5 p.m.

Day 3, 9 a.m.: Set up temperature control on the measurement tempering blocks. At 9 a.m. the samples will have completed their 40-hr cycle and can be transferred to 0°C until 10:30 a.m. They are then held at the measurement temperature and can be measured starting at 11:30 a.m. It should be possible to complete the measurements and make the calculations by noon and then prepare an additional batch in the afternoon. It is also possible to have another set of samples prepared and measured on even-numbered days.

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Key References

Firestone, 1998. See above.

A detailed step-by-step guide to the methods described here and the important alternative dilatometry.

Lawler and Dimick, 1998. See above.

Excellent review of the physics of fat crystallization useful in interpreting the results of these measurements.

O'Brien, 1998. See above.

Chapter 3, Fats and Oils Analysis, contains a lot of practical information on the methods and a useful comparison of NMR, DSC, and dilatometry.

Internet Resources

http://www.aocs.org/

The American Oil Chemists' Society Web site contains several links of interest.

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Lipid Crystal Characterization

The structure (e.g., number, size, distribution) of fat crystals is difficult to analyze by common microscopy techniques (i.e., electron, polarized light), due to their dense and interconnected microstructure. Images of the internal structures of lipid-based foods can only be obtained by special manipulation of the sample. However, formation of thin sections (polarized light microscopy) or fractured planes (electron microscopy) still typically does not provide adequate resolution of the crystalline phase. Confocal laser-scanning microscopy (CLSM), which is based on the detection of fluorescence produced by a dye system when a sample is illuminated with a krypton/argon mixed-gas laser, overcomes these problems. Bulk specimens can be used with CLSM to obtain high-resolution images of lipid crystalline structure in intricate detail.

This unit describes the analysis of lipid crystal microstructure using CLSM. A combination of Nile Red and Nile Blue dyes is dissolved in the melted samples. Nile Red is very fluorescent and allows the use of the laser source at ≤10% power, which prevents the dyes from burning or samples from melting. Moreover, crystal structure is well defined and air bubbles are easy to distinguish from crystals. Nile Blue is necessary to better distinguish the background from crystals. This lipophilic stain diffuses into the oil phase of a sample and generates a deep yellow fluorescence, whereas the solid fat does not fluoresce. The combination of dyes described here should be suitable for most fat systems. Sample preparation and image collection are discussed in detail. Familiarity with CLSM techniques is required (see, e.g., Smith, 1998).

This protocol is written primarily for samples where the dyes can be added into the molten fat, which can then be observed either in the molten state, if a hot substage is attached to the microscope stage, or after allowing it to solidify. To obtain the best images, dyes should be dissolved in the fats before samples are crystallized.

Materials

Nile Red (Sigma)
Nile Blue, sulfate salt (Sigma)
Fat sample

Ovens, 60° and 80°C
Razor blade or microtome (optional)
Confocal microscope and appropriate fluorescence filter (e.g. Bio-Rad MRC 600 or MRC 1024)

Crystallize fat sample

1. Weigh out 0.5 mg Nile Red and 100 mg Nile Blue.

   It is important to weigh Nile Red accurately. Nile Red modifies lipid nucleation kinetics when used at higher concentrations and, thus, modified crystal images may be obtained if the concentration is too high. The concentration recommended in this protocol has been proven not to modify lipid crystallization kinetics.

2. Melt 500 g fat sample in a beaker and hold at 80°C for 30 min.

   This procedure will destroy all existing crystals. This temperature and time will be sufficient for most fats.

3. Add dyes to hot sample. Hold sample in an oven at 60°C for ≥5 hr to dissolve dyes.

   This step is crucial to obtain high-quality images. If enough dye is not dissolved, images will be very poor. Nile Red is very soluble in fats, but Nile Blue is hard to dissolve. It takes

Contributed by M.L. Herrera and R.W. Hartel
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≥5 hr to dissolve the dye. The sample can be kept at 60°C overnight to ensure full dissolution. The sample should not be stirred, as this may encourage oxidation.

The fats used to formulate products such as margarine or shortening do not crystallize at 60°C, but if a fat sample does crystallize at this temperature, a higher temperature can be used. It is better to use as low a temperature as possible to avoid oxidation.

4. Apply the time-temperature profile necessary to crystallize the sample.

Different structures will be obtained according to the processing conditions selected. Cooling and agitation rates should be controlled accurately to obtain good repeatability. Crystallization temperature is another crucial parameter (see Critical Parameters and Troubleshooting for further discussion).

During experiments the authors controlled crystallization temperature with an accuracy of ± 0.2°C/min. For cooling rates, a difference of 2°C/min may be significant depending on the temperature range and type of fat. Agitation rates that differ by 50 rpm produce significant differences in crystal size.

Prepare for observation

5. Prepare duplicate or triplicate ~2- to 3-mm-thick sections of the sample on a microscope slide. For best results, allow sample to solidify directly on the slide so that the surface is sufficiently smooth for imaging. If starting with solidified products, prepare section with a razor blade or a microtome, if possible, for optimal smoothness.

Samples that are 2 to 3 mm thick are suitable for imaging by CLSM and work well for products with solid fat content (crystal phase volume; UNIT D3.1) between ~10% and 50%. Fats with higher solid content must be thinner to allow sufficient light penetration. This method does not work well for samples with very high (>75% to 80%) solid fat content, as the samples are generally too opaque to allow sufficient light penetration.

6. Select an appropriate fluorescence filter for a confocal microscope.

Filter selection depends on the dye system. Incorrect filter selection leads to poor images. The appropriate fluorescent light wavelength for this mix of Nile Red and Nile Blue is 488 nm.

7. Set laser power at 10%.

Setting the laser source at 10% power prevents the dyes from burning and the samples from melting.

8. To obtain a high-quality image, set the pinhole value as close to zero as possible.

The pinhole value sets how much out-of-focus light is allowed to reach the detector. For dark samples it is sometimes not possible to close the pinhole. In this case, use position 2 and give more light to the image with the gain bottom of the microscope.

9. To select the proper objective, start observing sample with the lowest magnification.

Lower magnification gives a better idea of the total sample, especially if crystals are large. Higher magnification allows observation of the details.

Analyze sample with Z-series images

10. Set microscope depth to zero.

This corresponds to the focal plane, which produces the most brilliant image.

11. Move the microscope stage to obtain the lowest image available for the sample. When image becomes too dark, set this depth as the lowest depth.

12. Repeat the same procedure while moving the microscope stage to obtain the highest-level image.

Z-series images allow samples to be studied at different depths, and thus provide information about the three-dimensional distribution of solids and liquid. The number of fields (z series) that can be viewed depends on pinhole position, objective, and sample brightness. Fewer planes can be observed in darker samples. Images taken at steps of ~3μm are needed to obtain a three dimensional representation of the microstructure.

13. Save electronic files of images and use commercial software to analyze crystal size, size distribution, and number.

Quantitative analysis of these images is accomplished in the same manner as for any microscope image, depending on the detail of analysis desired. Automated image analysis software can be used to provide a variety of information. Crystal phase volume (solid fat content; UNIT D3.1) can be obtained by counting dark pixels (according to some empirical threshold factor).

COMMENTARY

Background Information

Light microscopy is a well-developed and increasingly used technique for studying the microstructure of food systems in relation to their physical properties and processing behavior. Good-quality, high-resolution images of the internal structures of foods can only be obtained from thin sections of the sample. Procedures that apply substantial shear and compressive forces may destroy or damage structural elements, and sectioning is time consuming and involves chemical processing steps that may introduce artifacts and make image interpretation difficult. Confocal laser-scanning microscopy (CLSM) overcomes these problems. In this instrument, image formation does not depend on transmitting light through the specimen and, therefore, bulk specimens can be used for the first time in light microscopy. The instrument uses a focused scanning laser to illuminate a subsurface layer of the specimen in such a way that information from this focal plane passes back through the specimen and is projected onto a pinhole (confocal aperture) in front of a detector. Only a focal plane image is produced, which is an optical slice of the structure at some preselected depth within the sample. By moving the specimen up and down relative to the focused laser light, a large number of consecutive optical sections with improved lateral resolution (compared with conventional light microscopy) can be obtained with a minimum of sample preparation.

CLSM is a relatively new method in food science. Several reviews discussing the application of this technique in microstructural studies of food products have been published recently (Heertje et al., 1987; Blonk and Vanaalst, 1993; Marangoni and Hartel, 1998). These reviews have shown the advantages of using CLSM over conventional techniques for studying the relation between the composition, processing, and final properties of these products.

Comparatively few papers have yet been published dealing with food-related topics. A technique to study the stability of food emulsions, an important aspect of the processing and shelf-life of many food systems, was developed by Heertje et al. (1990). The cellular structure of selected apple varieties was observed by CLSM (Lapsley et al., 1992), as was the effect of processing on the structural organization of cereal grain (Yiu, 1993). The fractal structure of the aggregates in food protein gels (Hagiwara et al., 1997) and the microstructure of acid milk gels as affected by fat content and heat treatment (Lucey et al., 1998) were also investigated by this technique.

Critical Parameters and Troubleshooting

As mentioned above, this protocol is written primarily for samples where the dyes can be added into the molten fat; the sample can then be observed either in the molten state or after allowing it to solidify. To obtain the best images, dyes should be dissolved in the fats before samples are crystallized. This technique can also be used for solidified lipid-based products, although the dyes must be diffused into the sample from the surface. In margarine, shortenings, or solid food, the dye can be applied directly to the surface of the specimen as a fine dusting of powder, as a solution in oil, or in a mixture of polyethylene glycol and glycerol followed by the application of a coverslip (Brundrett et al., 1991). For solid foods, such preparations need to be left for some hours before being examined to allow the dye sufficient time to diffuse into the matrix. A good image is only obtained if the dye and food molecules are brought into molecular contact.

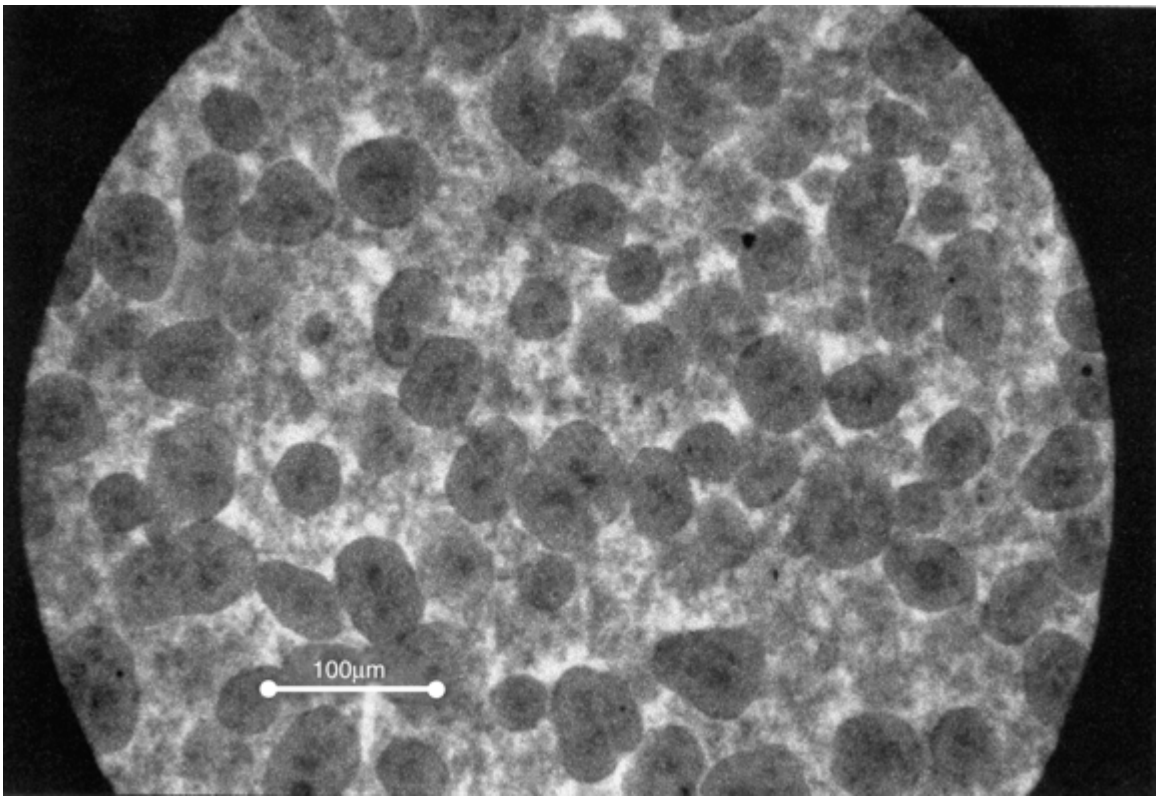

Figure D3.2.1 Confocal image of a mixture of equal parts high-melting and low-melting fractions of milk fat crystallized at 27.5°C with rapid (5.5°C/min) cooling and an agitation rate of 150 rpm.

The principal cause of poor image quality using CLSM is poor dissolution of the dyes, especially Nile Blue. In the method as described, Nile Red co-crystallizes with the fat crystals and, thus, a higher resolution of crystal surface is obtained. However, it is difficult to distinguish solid from liquid fat with only Nile Red. Nile Blue is necessary because it is dissolved in the liquid phase and provides fluorescence for contrast. As a result, the quality of images is very much improved. The disadvantage of Nile Blue is its low solubility. Special attention should be paid to sufficiently dissolving the dye at 60°C.

Crystallization behavior of fats is determined by the chemical composition of the fat and by the processing conditions selected. Cooling rate influences the morphology and size of the crystals obtained. In many fats, it also determines the polymorphic form in which the fat crystallizes. Agitation rate and crystallization temperature have a marked influence on crystal size. Shape, size, and size distribution of crystals, as well as interactions between crystals, determine the rheology of a lipid-based product (e.g., butter, margarine). Large crystals that form strong links give a more solid-like behavior, whereas smaller crystals formed by less material give a lower elastic modulus that results in a more viscous behavior. The required kind of crystals depends on the desired product characteristics. In margarine, small crystals that form a network able to retain liquid oil and that do not provide a sandy texture are required. For other products, such as dressings, larger crystals are required. Food texture is not only related to the solid content but also to the different crystalline microstructures of fats. Special attention should be paid to the selection and control of processing conditions to ensure repeatable crystalline microstructures.

Anticipated Results

Excellent agreement with solid fat content as measured by pulsed NMR (*UNIT D3.1*) has been found using this method. Crystal size, number, and distribution may also be obtained from automated image analysis software, although the accuracy of this measurement will depend on the nature of the lipid crystal structure. Samples with a high degree of network formation will be difficult to analyze quantitatively. By accumulating sequential *z*-series images, three-dimensional representations of the crystalline microstructure can be obtained. Poly-

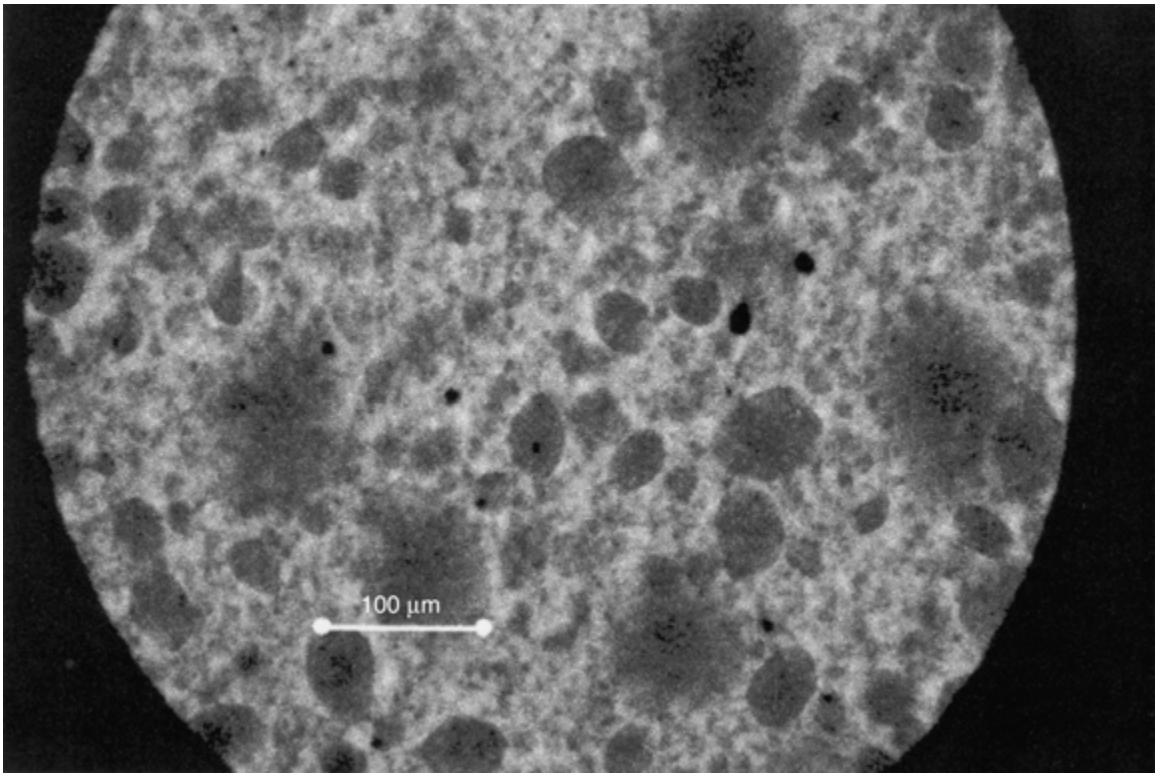

Figure D3.2.2 Confocal image of a mixture of equal parts high-melting and low-melting fractions of milk fat crystallized at 27.5°C with slow (0.2°C/min) cooling and an agitation rate of 150 rpm.

morphic forms are better distinguished by X-ray crystallography. The same polymorphic form can give different structures, and different polymorphic forms can give very similar structures. CLSM provides information about the way solid and liquid phases are distributed, and structures obtained by CLSM allow some prediction of rheology but not polymorphism.

Figure D3.2.1 shows the image obtained when a mixture of equal parts high-melting fraction (Mettler dropping point 46.5°C) and low-melting fraction (Mettler dropping point 16.5°C) of milk fat was crystallized to 27.5°C at a cooling rate of 5.5°C/min and an agitation rate of 150 rpm. Fat crystals are dispersed in a liquid phase. Air bubbles can be observed as very black spots, generally on the surface of crystals. Figure D3.2.2 shows an image of the same sample crystallized at 0.2°C/min. These figures show the effect of cooling rate on crystal structure. At a slow cooling rate, crystals were more dense and ill defined; at a fast rate, crystals had a more regular shape and a more homogeneous size.

Time Considerations

Samples should be prepared the day before analysis, because dissolving dyes requires that they be placed in an oven ≥5 hr or overnight. It takes ~30 min to collect a series of images at 3-µm intervals and 25 depths.

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A detailed description of the confocal microscope, basis of this technique, selection of dyes, and application in food science is described.

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Emulsion Droplet Size Determination

Quality attributes of food emulsions, such as appearance, stability, and rheology, are strongly influenced by the size of the droplets that they contain (Friberg and Larsson, 1997; McClements, 1999). For example, the creaming stability of an emulsion decreases as droplet size increases. Analytical techniques that provide quantitative information about droplet size are therefore required to aid in the development and production of high-quality emulsion-based food products. A variety of analytical techniques have been developed to measure droplet size, e.g., laser diffraction, electrical pulse counting, sedimentation techniques, and ultrasonic spectrometry (McClements, 1999). These techniques are used for fundamental research, product development, and quality assurance. This unit focuses on the two most commonly used techniques in the food industry, laser diffraction and electrical pulse counting.

LASER DIFFRACTION DETERMINATION OF DROPLET SIZE DISTRIBUTION

BASIC PROTOCOL 1

This protocol describes a laser diffraction technique used to measure the droplet size distribution of emulsions. A monochromatic beam of laser light is transmitted through a dilute emulsion and the resulting diffraction pattern is measured using a series of light-sensitive detectors (Fig. D3.3.1). The diffraction pattern is the result of scattering of the laser beam by the droplets in the emulsion being analyzed, and its precise form depends on the droplet characteristics (i.e., size, concentration, and complex refractive index). Information about droplet size distribution and concentration is determined by finding the values that give the best agreement between the measured diffraction pattern and that predicted by light-scattering theory. This theory assumes that each photon of light is only scattered by a single droplet, which means that the emulsion must be dilute (<0.1% droplets) to avoid multiple scattering. Most commercially available instruments can measure droplet diameters between 0.1 and 1000 μm, although some have special features that enable them to analyze smaller droplets. The precise operating procedure for a particular laser diffraction instrument depends on its design, which varies between manufacturers. This protocol is, therefore, fairly general, and the laboratory manuals of specific instrument manufacturers should be consulted for a more detailed description.

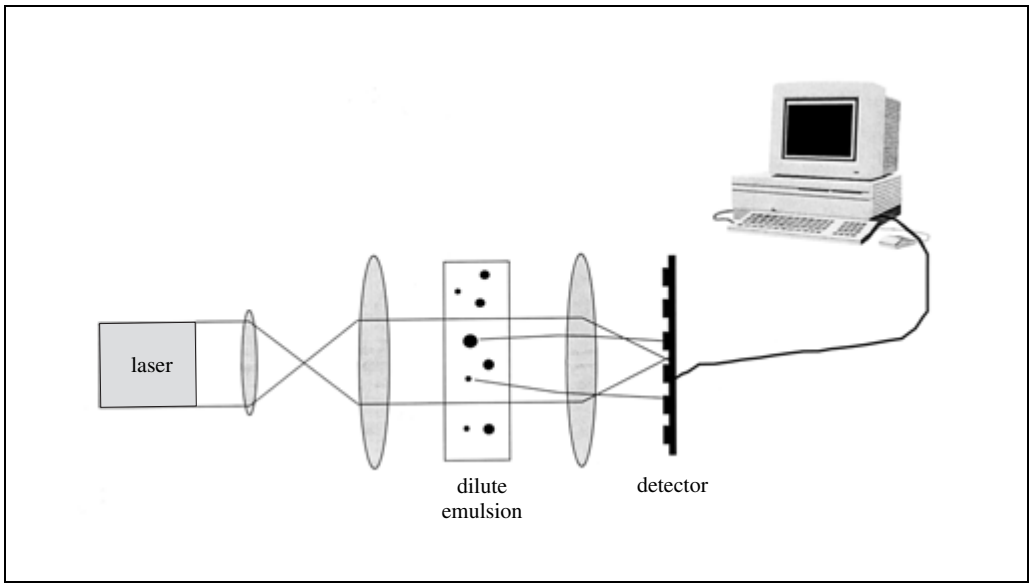

Figure D3.3.1 Schematic diagram of laser diffraction instrument for determining droplet size distribution.

Contributed by D. Julian McClements
Current Protocols in Food Analytical Chemistry (2001) D3.3.1-D3.3.9

In the near future, an ISO standard for the installation and validation of laser diffraction units will be published.

Materials

Distilled water or buffer solution (see recipe)
Sample emulsion
Calibration standard containing particles of known diameter (see recipe)
Laser-diffraction instrument designed for particle size analysis (e.g., Mastersizer, Malvern Instruments)

1. Turn on the laser diffraction instrument and allow it to warm up for 30 min before taking measurements.

   This gives the laser enough time to reach a consistent and reliable output.

2. Zero the instrument by measuring the background diffraction pattern of distilled water or buffer solution.

   Most commercial instruments display the variation of light intensity with scattering angle. This should be fairly flat across the range of scattering angles where the measurements are made (except at smaller angles where the laser beam directly passes through the sample). If it is not flat, there may be problems with a dirty cuvette, insufficiently clean solution, air bubbles in the solution, or incorrect alignment of the cuvette (see Troubleshooting).

3a. *If sample emulsion has a droplet concentration within the measurement range of the instrument:* Analyze directly by replacing the distilled water or buffer solution with sample, and measuring its diffraction pattern.

   The measurement range of an instrument depends on a number of instrumental and sample factors and must be determined for each system. Most commercial instruments have an indicator that shows when the droplet concentration is in the appropriate measurement range.

3b. *If sample emulsion is too concentrated to analyze directly:* Dilute prior to analysis according to the following criteria.

   a. If only the droplet size distribution is required, then dilute by placing a sufficient number of drops of emulsion into the distilled water or buffer solution to reach the optimum transmittance range for the instrument (which is normally indicated on a computer screen). Stir the sample for ~1 min to ensure homogeneity and then measure the diffraction pattern.

   b. If both the droplet size distribution and concentration of the emulsion are required, and the instrument is capable of measuring both, then dilute the emulsion to a concentration that is within the optimum transmittance range of the instrument using a known amount of distilled water of buffer solution. Place the diluted emulsion in the instrument and measure the diffraction pattern.

      Once the droplet concentration of the diluted emulsion has been measured, it is possible to determine the droplet concentration of the initial emulsion: $\phi_I = \phi_D V_I/V_D$, where ϕ is the droplet volume fraction, V is emulsion volume, and the subscripts I and D refer to the initial and diluted emulsions, respectively.

4. Obtain results from instrument.

   After the diffraction pattern from the sample emulsion is measured, it is automatically corrected by the instrument using a background-subtraction routine to account for any extraneous scattering from the solvent or imperfections in the optical system. The instrument automatically calculates the droplet size distribution (and sometimes the droplet concentration) that gives the best agreement between the measured diffraction pattern and that predicted by light-scattering theory. Finally, the results are presented as a table and/or graph.

Be sure to use the correct values for the refractive index and absorptivity of the droplets and continuous phase in the mathematical analysis. These can usually be found in the literature obtained from the instrument manufacturer, or are measured in the laboratory.

5. Periodically, ensure that the instrument is working correctly by measuring the particle size distribution of a calibration standard and checking it against its known particle size.

   Again it is important to use the correct refractive index and absorptivity of the particles and continuous phase in the mathematical analysis. (see Critical Parameters).

ELECTRICAL PULSE COUNTING DETERMINATION OF DROPLET SIZE DISTRIBUTION

ALTERNATE PROTOCOL 1

This protocol describes an electrical pulse counting (also called "electrozone sensing") technique used to measure the droplet size distribution of emulsions. These instruments have been commercially available for many years and are widely used in the food industry (McClements, 1999). The emulsion to be analyzed is diluted in a weak electrolyte solution, which is placed in a beaker with two electrodes dipped into it (Fig 3.3.2). One of the electrodes is contained in a glass tube that has a small aperture in it, through which the emulsion is drawn (Hunter, 1986; Mikula, 1992; Lines, 1996). When an oil droplet passes through the aperture it causes a decrease in the current between the electrodes because oil has a much lower electrical conductivity than water. Each time a droplet passes through the aperture, the instrument records a decrease in current which it converts into an electrical pulse. The instrument controls the volume of liquid that passes through the aperture; therefore, droplet concentration can be determined by counting the number of electrical pulses in a known volume.

Most commercially available instruments can measure droplet diameters between 0.4 and 1200 μm, although a number of tubes with different-sized apertures are required to cover the whole of this range (Lines, 1996). The measured droplet diameter is precise, i.e., better than 1%. The operating procedure for a particular instrument depends on its design, which varies between manufacturers. The protocol given below is therefore fairly general, and the laboratory manuals of specific instrument manufacturers should be consulted for a more detailed description.

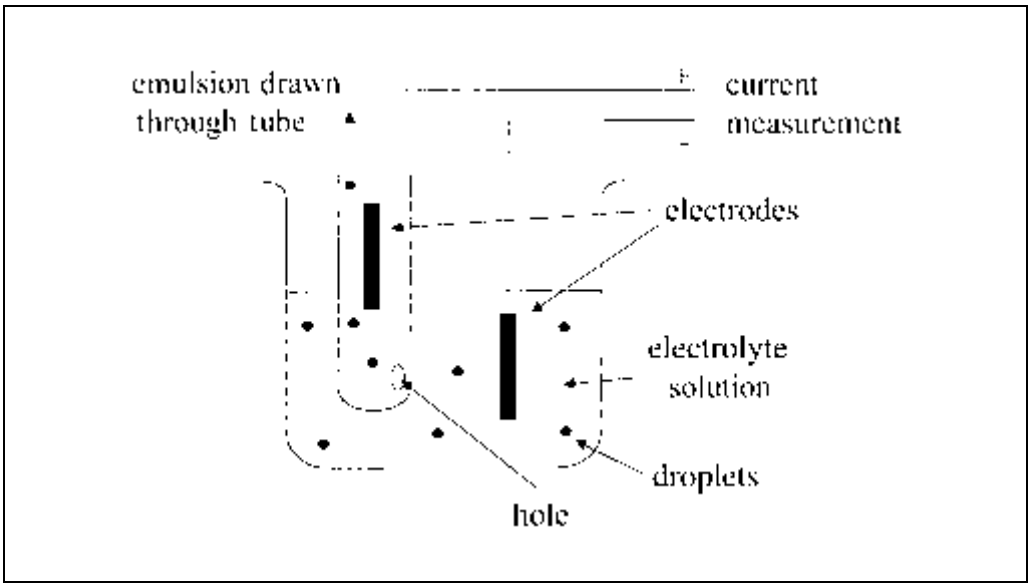

Figure D3.3.2 Schematic diagram of electrical pulse counting instrument for determining droplet size distribution.

Materials

Calibration standard containing particles of known diameter (see recipe)
Electrolyte solution (see recipe)
Sample emulsion

Electrical pulse counting instrument (e.g., Coulter Counter, Beckman Coulter)
Measurement cell with appropriate aperture for sample being analyzed

1. Turn on the instrument and allow it to warm up for 30 min before taking measurements.
2. Calibrate the instrument as follows.
   a. Fill the measuring cell with particle size calibration standard (electrolyte solution containing an appropriate concentration of suspended particles).
   b. Turn on a stirrer to ensure homogenous distribution of the particles within the measurement cell.
   c. Measure the pulse height of a large number of particles and then calculate the calibration factor, k (= d^3/P); these procedures are done automatically by the instrument.

      The instrument only needs to be calibrated periodically, but it is useful to always analyze a calibration standard before making a set of measurements to ensure that the instrument is performing correctly.
3. Dilute the emulsion to be analyzed in electrolyte solution to obtain a final droplet concentration less than ~10^5 droplets/ml.

   The instrument automatically pulls the particles through the aperture and measures the pulse height (P) and number of pulses per unit volume of a large number of particles (~10,000). It uses this information to calculate the particle size distribution (d^3 kP) and number of particles per unit volume. This information is reported to the user in the form of a table or a plot.
4. After the analysis has been completed, remove the emulsion and thoroughly clean the measurement cell with electrolyte solution.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. If necessary filter the water before use to remove any particulate matter e.g., using a 0.22 or 0.44 μm filter.

Buffer solution (laser diffraction)

The composition of the buffer solution used to dilute an emulsion depends on the characteristics of the emulsion. To avoid changes in the aggregation of emulsion droplets, it is best to use a buffer solution with the same pH and composition as the continuous phase of the emulsion. To prevent droplet aggregation, it may be necessary to incorporate a small amount of surfactant in the buffer solution.

Electrolyte solution (electrical pulse counting)

The droplets being analyzed must normally be suspended in an electrolyte solution to increase the flow of current between the electrodes. Commonly used electrolyte solutions are 0.85% (w/v) NaCl or 5% (w/v) trisodium orthophosphate. The electrolyte solution should be free from any particulate matter and be chemically compatible with the sample.

Particle size calibration standard

Spherical particles of known diameter (e.g., ~5% to 20% of the diameter of the aperture in the glass tube) are used to calibrate the electrical pulse counting instrument. The particles are suspended to an appropriate concentration in electrolyte solution (see recipe). Monodisperse latex particles are commercially available, which can be used for this purpose. Particle size calibration standards can be obtained from a number of chemical suppliers or from the National Institute of Standards and Technology (e.g., NBS 1003b). Lines (1996) lists a number of standards that are appropriate for this purpose.

COMMENTARY

Background Information

Laser diffraction

Laser diffraction is the most commonly used instrumental method for determining the droplet size distribution of emulsions. The possibility of using laser diffraction for this purpose was realized many years ago (van der Hulst, 1957; Kerker, 1969; Bohren and Huffman, 1983). Nevertheless, it is only the rapid advances in electronic components and computers that have occurred during the past decade or so that has led to the development of commercial analytical instruments that are specifically designed for particle size characterization. These instruments are simple to use, generate precise data, and rapidly provide full particle size distributions. It is for this reason that they have largely replaced the more time-consuming and laborious optical and electron microscopy techniques.

Laser diffraction is most suitable for analyzing dilute emulsions that are fluid, and therefore competes directly with electrical pulse counting methods, which are applicable to similar systems (see Alternate Protocol). Most laser diffraction instruments can cover a wider range of particle sizes (i.e., 0.01 to 1000 μm) than electrical pulse counting instruments (i.e., 0.4 to 1000 μm using a number of different aperture sizes), and do not require the presence of electrolyte in the aqueous phase, which could destabilize some electrostatically stabilized emulsions. Nevertheless, electrical pulse counting techniques are considered to have greater resolution.

The accuracy to which the droplet size distribution of an emulsion can be determined by a properly functioning and correctly operated laser diffraction instrument depends upon two major factors: (1) the design of the optical system used to measure the diffraction pattern resulting from the transmission of a laser beam through the cuvette; and (2) the sophistication of the mathematical model used to convert the measured diffraction pattern into a droplet size distribution. The number, position, and quality of the detectors used to measure the angular dependency of the laser beam determine the accuracy to which the diffraction pattern can be measured. The greater the number of detectors, the wider the range of angles covered, and the greater the sensitivity of the detectors, the more accurately the diffraction pattern can be determined. More sophisticated instruments, which are normally only used in research laboratories, have a detector that can be positioned at any angle to the laser beam.

The droplet size distribution and concentration are determined by finding the values that give the best agreement between the measured diffraction pattern and that predicted by light scattering theory. The most rigorous light scattering theory, called the Mie theory, is applicable to suspensions containing any droplet size and refractive index (Kerker, 1969; Farinato and Rowell, 1983). Older particle sizing instruments took a considerable amount of time to determine the droplet size distribution because a great deal of computation was required. Consequently, a number of instrument manufacturers utilized simpler approximations to the Mie theory that were limited to certain particle size ranges (Kerker, 1969). The use of these theories reduced the required computation time, but sacrificed the accuracy of the results. Another method of reducing the computation time was to assume that particle size distribution followed a certain mathematical form (e.g., normal or log-normal); however, this also led to a reduction in accuracy. Recent advances in electronics and computers have led to the availability of modern instruments that can rapidly solve the Mie theory without requiring any simplifying assumptions. Consequently, these instruments have a greater accuracy than their older counterparts.

Table D3.3.1 Representative Refractive Indexes for Common Lipids Measured at Sodium D Line

Decane	1.411
Dodecane	1.422
Tetradecane	1.429
Hexadecane	1.434
Corn oil	1.475
Olive oil	1.466
Canola oil	1.465
Soybean oil	1.474
Sunflower oil	1.469

For the reasons described above, the droplet size distribution of the same emulsion measured on different laser diffraction instruments can be significantly different, depending on the precise design of the optical system and the mathematical theory used to interpret the diffraction pattern. It should be noted, however, that the most common source of error in particle size analysis is incorrect operation of the instrument by the user. Common sources of user error are introduction of air bubbles into the sample, use of the wrong refractive index, insufficient dilution of emulsion to prevent multiple scattering, and use of an unclean optical system.

Electrical pulse counting

The electrical pulse counting principle was developed over 50 years ago by Wallace Coulter and was turned into a commercial instrument by the Coulter Corporation (Lines, 1996). The emulsion to be analyzed is placed in a beaker that has two electrodes dipped into it (Allen, 1990; Lines, 1996). One of the electrodes is contained in a glass tube that has a small aperture in it, through which the emulsion is sucked. When an oil droplet passes through the aperture it causes a decrease in the current between the electrodes because oil has a much lower electrical conductivity than water. Each time a droplet passes through the aperture, the instrument records a decrease in current that it converts into an electrical pulse. The instrument controls the volume of liquid that passes through the aperture, and so the droplet concentration can be determined by counting the number of electrical pulses in a known volume. When the droplets are small compared to the diameter of the aperture, the droplet size is simply related to the height of the pulses: $d^3 = kP$, where, d is the droplet diameter, P is the pulse height, and k is an instrument constant, which is determined by recording the pulse height of a suspension of monodisperse particles of known diameter.

To cover the whole range of droplet sizes from 0.4 to 1200 μm it is necessary to use glass tubes with different-sized apertures. Typically, droplets between about 2% and 60% of the diameter of the aperture can be reliably analyzed. Thus, a tube with a aperture of 16 μm can be used to analyze droplets with diameters between ~0.3 and 10 μm. If an emulsion contains a wide droplet-size distribution it may be necessary to use a number of glass tubes with different aperture sizes to cover the full size distribution.

The major disadvantage of the laser diffraction and electrical pulse counting techniques is that they are only directly applicable to dilute emulsions or emulsions that can be diluted without disturbing the particle size distribution. However, many food emulsions are not dilute and cannot be diluted, either because dilution alters the particle size distribution or because the original sample is partially solid. For concentrated systems it is better to use particle-sizing instruments based on alternative technologies, such as ultrasonic spectrometry or NMR (Dickinson and McClements, 1996).

Critical Parameters

Laser diffraction

For accurate measurements it is important to enter the correct optical constants of the dispersed and continuous phase into the theory used to calculate the droplet size distribution. In most instruments, it is necessary to enter the refractive index and absorptivity of the component phases at the appropriate wavelength of the laser. Significant errors in the measured droplet size distribution will occur if incorrect optical parameters are used, particularly if the

refractive index of the particles is close to that of the surrounding liquid, if the particle size is closer to the laser wavelength, or if one of the components absorbs light strongly at the laser wavelength. It should be noted that most textbook values of the real part of the refractive index have been measured at 584 nm, which may not be the same as the laser used in the light-scattering instrument. It is possible to obtain values for the refractive index and absorptivity of oils from particle sizing instrument manufacturers or from the literature, or by carrying out measurements in the laboratory. Refractive indices for common oils are shown in Table D3.3.1.

Electrical pulse counting

For accurate measurements it is important to use an electrolyte solution that does not contain extraneous particulate matter and that does not promote droplet aggregation. In addition, it is important to use an aperture that is appropriate for the range of particle sizes in the sample being analyzed. Typically, the diameter of the emulsion droplets should be between ~2% and 60% of the diameter of the aperture in order to obtain accurate measurements. If the particles cover a large particle size range, it may be necessary to use two or more tubes with different apertures, and overlap the results.

Troubleshooting

Laser diffraction

Problems with cuvettes. During measurements, sample emulsions are contained within quartz cuvettes with fixed path lengths (typically between 5 and 10 mm). It is extremely important that a cuvette be free from extraneous matter or imperfections that would interfere with the diffraction pattern from blanks or samples. The cuvette should be free from scratches, dirt, or air bubbles that scatter the laser beam. It should, therefore, be cleaned regularly using materials that will not scratch its surface. The problem of slight imperfections in a cuvette is largely overcome by running a blank and a sample, since the background diffraction pattern from the blank is subtracted from that of the sample. Nevertheless, this method will not give accurate measurements when excessive imperfections are present. Any air bubbles attached to the cuvette walls or distributed through a blank or sample will scatter light and therefore interfere with the analysis. Air bubbles can be removed by mild centrifugation or sonication of the liquid. Alternatively, they can be prevented from forming by avoiding excessive agitation during the preparation or stirring of the liquid to be analyzed. The presence of scratches, dirt, or air bubbles in a blank is often seen as a non-flat background in the diffraction pattern. In the absence of imperfections, the diffraction pattern should be approximately zero across the wider scattering angles.

The cuvette must be correctly placed in its holder so that the laser beam is perpendicularly incident upon it. If the cuvette is not fixed correctly the laser beam is deflected from its normal path, which leads to a nonuniform and incorrect diffraction pattern. Many commercial instruments have the ability to adjust the alignment of the laser beam once the cuvette has been correctly inserted into the sample holder, to ensure that it is perpendicularly incident.

Problems with droplet aggregation. Droplet size distributions of flocculated emulsions determined by laser diffraction should be considered as approximate. The theory used by commercial instruments to calculate the size distribution assumes that the particles are isolated homogeneous spheres with well-defined optical properties. In flocculated emulsions, the droplets aggregate into fairly loose, nonspherical and heterogeneous "particles," which have ill-defined optical properties. Thus the particle size distribution determined by light scattering is only an approximate indication of the actual size of the flocs. In addition, emulsions often need to be diluted (to eliminate multiple scattering effects) and stirred (to ensure they are homogeneous) prior to making measurements. Dilution and stirring could disrupt any weakly flocculated droplets, but leave strongly flocculated droplets intact. Thus, the measured particle size distribution will depend on the degree of dilution and mechanical agitation that the sample has experienced, as well as the characteristics of the solution used to carry out the dilution (e.g., pH, ionic strength, temperature). It is also important to choose an appropriate solution to dilute emulsions that are not flocculated in their concentrated state, but that may become flocculated if diluted using an inappropriate solution. As a rule of thumb, it is advisable to dilute an emulsion with a solution that has the same properties and composition as its continuous phase.

Extensive dilution of a surfactant-stabilized emulsion may cause some of the surfactant molecules to move from the droplet surface into the continuous phase. This process can cause the droplets in a diluted emulsion to become unstable and coalesce. For this reason, it is often

important to dilute the emulsions with a solution containing enough surfactant to prevent this displacement process from occurring—but not so much as to induce depletion flocculation or cause excessive foaming.

Electrical pulse counting

Blocked aperture. If the aperture is blocked with particulate matter, it can be removed by applying a slight back-pressure, by gently brushing the aperture, by soaking the aperture tube in acid, or by application of mild sonication.

Problems with droplet concentration. It is necessary to use emulsion concentrations that are sufficiently dilute to prevent two or more droplets passing through the aperture simultaneously; otherwise this will give particle sizes that are larger than expected. The upper particle concentration limit depends on the size of the aperture and the radius of the particles being examined (Lines, 1996). For example, the concentration should be less than ~10^5 particles/ml for a 100-μm aperture.

Problems with droplet aggregation. Droplet aggregation may interfere with the analysis of an emulsion in a number of different ways. If an operator is interested in measuring the size of the droplets in a nonaggregated emulsion, it is important to prevent aggregation from occurring during sample preparation and measurement. Aggregation could be promoted by the need to dilute emulsions in an electrolyte solution, because this can influence the electrostatic interactions between droplets. On the other hand, if the operator is interested in characterizing the degree of flocculation in an emulsion, it is important not to disturb the structure of flocs that were present in the original sample. Changes in floc structure may occur because of the electrolyte solution, dilution of the emulsion, or shearing of an emulsion by the stirrer in the device. It is important to be aware of these potential problems when designing an experiment, and to adjust the electrolyte type or concentration and stirring conditions accordingly.

Problems with large or dense particles. If the particles are particularly large or have a large density contrast with the surrounding medium, there may be problems due to creaming or sedimentation. In these situations, it is necessary to increase the viscosity of the suspending liquid and/or to use a stirring device to ensure that the particles are uniformly distributed (Lines, 1996).

Anticipated Results

Commercial particle sizing instruments based on laser diffraction and electrical pulse counting normally give out the droplet size distribution in the form of a table or plot. The table gives the droplet frequency (number or volume percentage) in different droplet size categories (radius or diameter). It is important to be clear whether the droplet frequency is being represented as the number or volume percentage, and whether the droplet size is represented by the radius or diameter, otherwise there may be considerable misunderstandings when comparing the data with that obtained by other groups. The plot may be presented as a histogram of droplet frequency versus droplet size category, or as a cumulative distribution representing the percentage of droplets below a specified size versus size (McClements, 1999). Some instruments are capable of giving the droplet concentration as well as the droplet size distribution

Time Considerations

Laser diffraction

If the optical properties of the component phases have already been entered into the instrument and the instrument has been blanked, a complete analysis takes less than 3 min to complete, from putting the sample into the measurement cuvette to obtaining the full particle size distribution. Blanking the instrument takes about the same time, and should be done regularly.

Electrical pulse counting

A complete analysis usually takes a few minutes to complete once the electrolyte solution has been prepared and the instrument is ready for operation.

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Kerker, 1969. See above.

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Thorough description of electrical pulse counting method.

van de Hulst, 1957. See above.

Standard text on theory of light scattering by colloidal suspensions

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Emulsion Stability Determination

An emulsion consists of two or more completely or partially immiscible liquids, such as oil and water, where one liquid (the dispersed phase) exists in the form of droplets suspended in the other (the continuous phase). Because the surface of each droplet is an interface between hydrophobic and hydrophilic molecules, it is inherently thermodynamically unstable. In addition, emulsion stability can be affected by a number of external (environmental) conditions (Figure D3.4.1). During normal storage of a food emulsion, a variety of chemical, physical, and microbiological stresses, in addition to temperature, will cause the destabilization of the emulsion over time (Figure D3.4.2). In this unit, two specific protocols are presented for determining the kinetic stability of emulsions. The first (see Basic Protocol 1) is used to give information about the long-term shelf life of a product, based on measurement of droplet size distribution and droplet concentration over the course of the intended storage time. The second (see Basic Protocol 2) describes a test used for emulsions that destabilize over short time periods (up to a week). As an alternative, a discussion of methods for altering the kinetics of emulsion destabilization and for using ultrasound or infrared waves to evaluate the emulsion is given (see Alternate Protocol). In addition, a method describing the measurement of droplet concentration is given (see Support Protocol). Droplet size distribution is measured as described in *UNIT D3.3*. The selected protocols do not require an extensive set of specialized equipment, with the single exception of a particle size analyzer. Standard laboratory equipment available in most laboratories should be sufficient to conduct emulsion stability measurements.

MONITORING DROPLET SIZE DISTRIBUTION AND CONCENTRATION DURING STORAGE

BASIC PROTOCOL 1

The so-called storage stability test is a standard test that is used across many different fields—e.g., the pharmaceutical, cosmetic, and food industries. The test is popular because it yields precise information about the long-term shelf life of emulsions. In this test, emulsions are stored under conditions that are applicable to those encountered in the actual production/consumption situation. It should be noted that the presented test protocol is time consuming and requires sampling over an extended period of time. In the

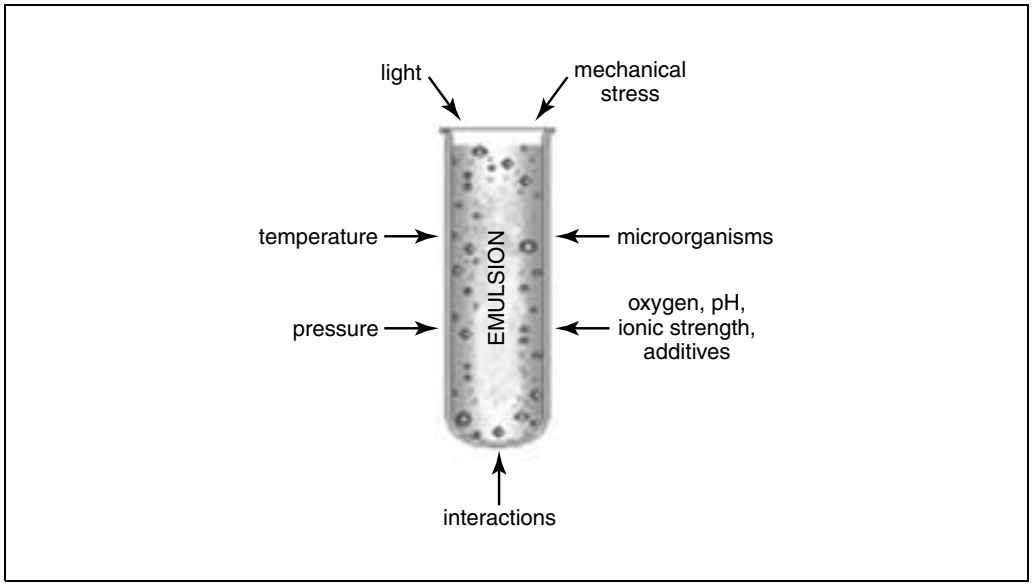

Figure D3.4.1 Various extrinsic parameters that can negatively impact the stability of emulsions.

Contributed by Jochen Weiss
Current Protocols in Food Analytical Chemistry (2002) D3.4.1-D3.4.17

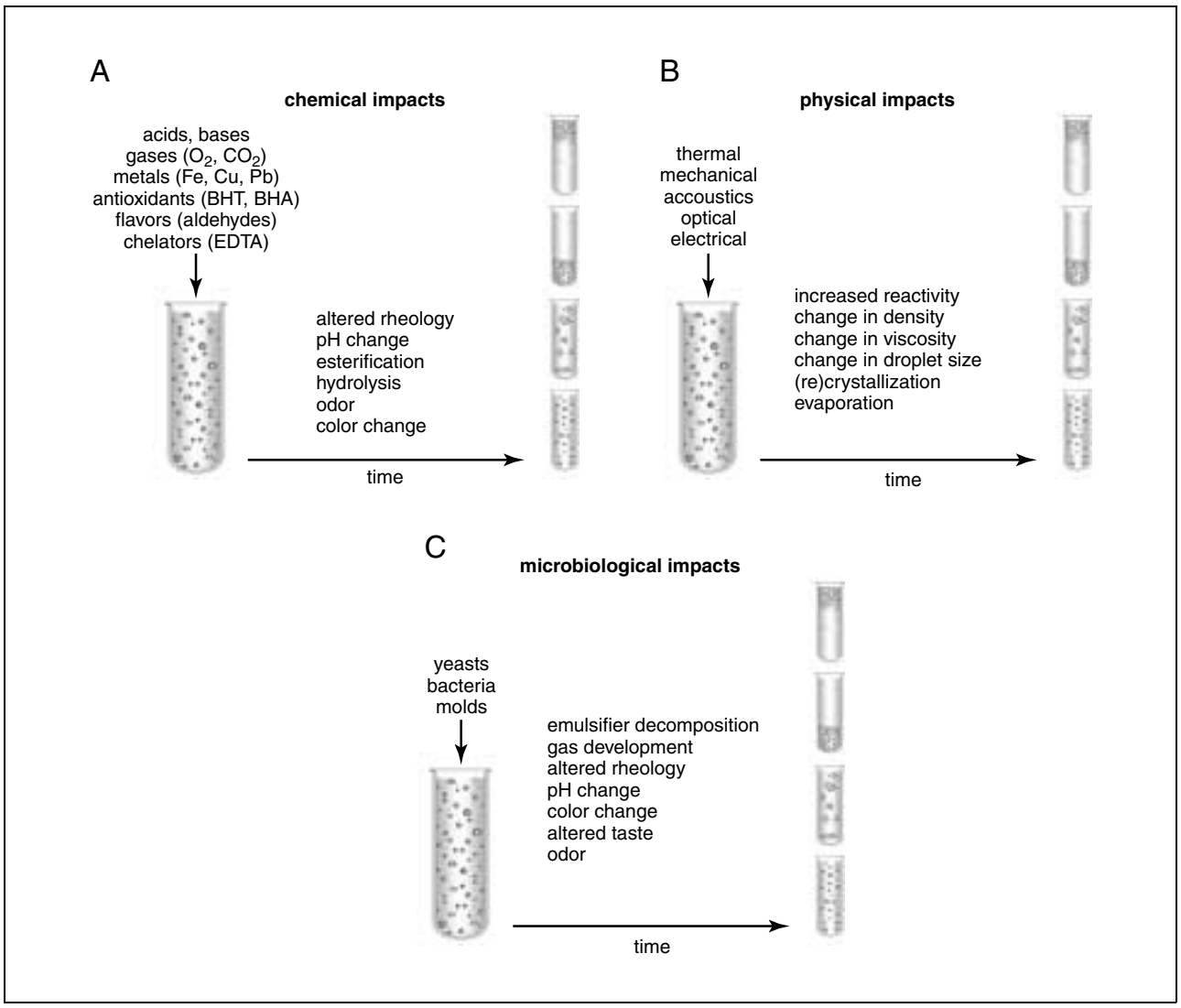

Figure D3.4.2 Chemical (**A**), physical (**B**), and microbiological (**C**) stresses result in the modification of colloidal and macroscopic properties, thus destabilizing the emulsion.

pharmaceutical industry, for example, these tests are often conducted over a period of more than a year. Because of this, a storage stability test should only be planned in the final stage of product development. Most importantly, selected sample emulsions should be representative of the entire batch. It is advisable to run side-by-side replications to ensure good data collection. A standard emulsion that has known stability behavior should be used as a control. Despite the mentioned disadvantages, storage stability tests are reliable and offer high accuracy. Hence, they are often unavoidable and should be an essential part of the quality assurance program of emulsion manufacturers.

Before performing the experiment, an appropriate storage container and facility should be chosen. A suitable storage container is required to hold the sample during the experiment. Figure D3.4.3 shows an example of a typical sample container. The glass beaker is equipped with two septum ports through which samples can be withdrawn by vertically introducing a needle attached to a syringe. The container should be at least 150 mm in diameter and 200 mm in height, with the lower port being located at ~30 mm and the upper port located at 150 mm. This minimizes errors that are introduced as the sample volume changes. As samples are withdrawn, the total emulsion volume decreases and the meniscus drops while the two sample ports remain at a fixed location. If the sample

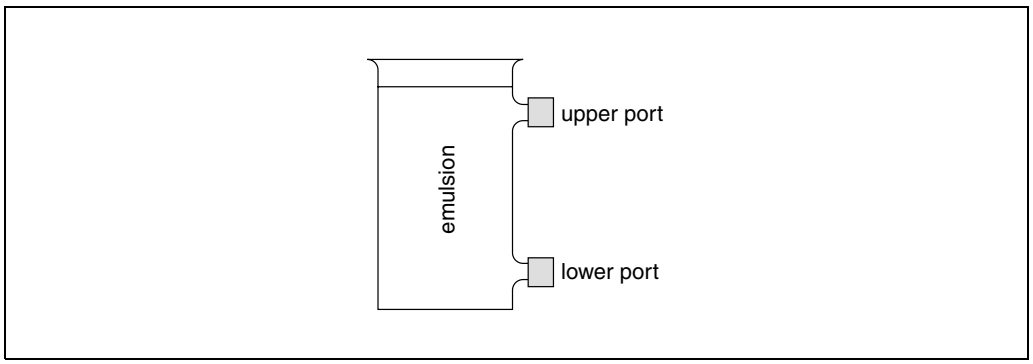

Figure D3.4.3 Suitable storage container for emulsion stability tests to determine changes in droplet size distribution and concentration during storage.

required to determine the particle size and concentration is large, the container size should be scaled up. For example, if the oil droplet concentration in the sample emulsion is very small, a larger volume may be required to accurately determine the droplet size. The choice of a particular protocol to measure the oil droplet concentration of emulsions—i.e., the use of a specific gravity bottle (see Support Protocol) versus the use of a digital density meter—may influence the sample volume as well.

Materials

Sample container (e.g., Fig. D3.4.3)
Sample emulsion
Syringe and needle

Additional reagents and equipment for measuring droplet size distribution (*UNIT D3.3*) and droplet concentration (see Support Protocol)

1. Prepare a sampling schedule using the total length of the planned experiment, which is based on the specific research or manufacturing needs (i.e., how long the emulsion needs to be stable for a particular application). Divide the experimental period by the total number of samples to obtain sampling intervals with a linear progression.

   For accurate stability evaluations, at least 10 to 12 samples should be withdrawn.

   A logarithmic time interval may be chosen if the emulsion consists of an oil that has a substantial solubility in the aqueous phase (e.g., aromatic or flavor oils; see Background Information, discussion of Ostwald ripening).

   An example of a suggested sampling schedule for a period of 30 days and a total of 15 samples is shown in Table D3.4.1.

2. Fill the sample container with sample emulsion and seal hermetically.

   Containers that are not properly sealed are prone to accelerated loss of continuous phase molecules due to evaporation. This in turn increases oil droplet concentration, which may subsequently accelerate emulsion breakdown because of the increase in the frequency of droplet collisions.

3. Transfer to the storage facility. Monitor and record environmental conditions in the storage chamber.

   Storage conditions should accurately reflect the conditions encountered in the actual production/storage process. The primary parameter to control is temperature. If the container is not properly sealed, other parameters such as relative humidity, air pressure, and air composition may affect the rate of emulsion breakdown.

4. Withdraw sample from the upper and lower ports of the sample container in intervals specified in the sampling schedule using an appropriately sized syringe and needle.

   The size of the syringe should be selected so that enough material can be withdrawn in one single step to allow accurate determination of both droplet size and concentration. Although the vertical introduction of the needle prevents undesirable horizontal mixing, multiple withdrawals of sample may affect the population that is present within each vial.

   In the pharmaceutical industry, it is common to immediately suspend a portion of sample in solutions of a small-molecule surfactant. The surfactant is expected to rapidly adsorb at incompletely covered droplet surfaces to prevent droplet coalescence between sample withdrawal and analysis of droplet size or concentration. However, the addition of small surfactant molecules can result in a displacement of the original emulsifier from the droplet interface and profoundly alter droplet-droplet interactions. Changes in system composition may therefore lead to greater errors than those generated by the lag between sample withdrawal and analysis (see Background Information, discussion of Ostwald ripening).

5. Measure droplet size distribution (*UNIT D3.3*) and droplet concentration (see Support Protocol). Record results on sampling schedule and recording form (Table D3.4.1).

   For an example using this analysis, see Anticipated Results.

Table D3.4.1 Typical Example of a Sampling Schedule and Reporting Form for Storage Stability Tests

	Day 0	Day 2	Day 4	…	Day 30
Droplet size (μm)	Cumulative volume distribution (%)[a]				
Upper port:					
0.1	1				
0.2	25				
0.5	50				
1	75				
2	100				
5	100				
10	100				
20	100				
50	100				
100	100				
Mean size	0.5				
Concentration					
Lower port:					
0.1	1				
0.2	25				
0.5	50				
1	75				
2	100				
5	100				
10	100				
20	100				
50	100				
100	100				
Mean size	0.5				
Concentration					

[a]See Basic Protocol 1, step 7 for the appropriate equation.

MEASURING DROPLET CONCENTRATION OF EMULSIONS

SUPPORT PROTOCOL

The protocol described here is based on the relationship between the density of the dispersed phase, the density of the continuous phase, the emulsion density, and the volume fraction, which is a measure of the oil droplet concentration. An essential part of the measurement is the precise determination of density. The method introduced below is the most inexpensive method to accurately determine density, but has the drawback that a relatively large sample volume is required.

If the determination of oil droplet concentration is a task that will be frequently conducted in the laboratory, the acquisition of a digital density meter is recommended. A digital density meter measures density and specific gravity with high precision within a short period of time. Digital density meters function based on the principle that a hollow glass tube vibrates with a specific frequency. This frequency changes when the tube is filled with sample. The higher the mass of the sample, the lower the frequency. The device measures the frequency and converts the signal into density. Digital density meters are usually equipped with a built-in thermostat unit that controls the temperature, so that no water bath is required.

NOTE: Small temperature fluctuations can result in large errors in calculated densities. The temperature of all materials (bottle and solutions) should therefore be carefully controlled during all stages of the procedure using either a temperature-controlled chamber or a water bath. If a water bath is used, all excess water must be wiped off the specific gravity bottle prior to measurements to prevent evaporative cooling effects and errors in weight determination.

Materials

Continuous phase
Dispersed phase
Sample emulsion
Specific gravity bottle with glass stopper
High-precision balance (±1 mg)

1. Measure the weight of an empty specific gravity bottle (m_b) using a high-precision balance.

   These bottles should preferably be used for oils and tars as specified in ASTM D 70/AASHTO T43.

   Depending on the viscosity of the emulsion under study, filling the bottle may be problematic. A number of different geometries are available for specific gravity bottles that are more or less suited for high- or low-viscosity fluids. A geometry should be chosen that allows the bottle to be completely filled without entrapping any air bubbles. The problem may be overcome by slightly increasing the temperature of the sample during the filling process; however, since volume usually decreases upon cooling the sample, additional material may have to be added to completely fill the bottle.

2. Fill the bottle with distilled deionized water and measure the total weight (m_t).

3. Calculate the volume of the bottle (V_g) as $V_g = m_w/\rho_w$, where ρ_w is the density of water at the specific measurement temperature (see Figure D3.4.4), and m_w is the weight of the water and is equal to $m_t - m_b$.

4. Fill the specific gravity bottle with continuous phase and measure the new weight of the filled bottle (m_t). Calculate the density of the continuous phase (ρ_c) as $\rho_c = (m_t - m_b)/V_g$.

5. Repeat with dispersed phase to determine its density (ρ_d).

Temperature (°C)	Density (kg/m^3)
5	0.99994
10	0.99969
15	0.99909
20	0.99819
25	0.99702
30	0.99561
35	0.99399
40	0.99217
45	0.99017
50	0.98799
55	0.98565
60	0.98316
65	0.98053
70	0.97775
75	0.97484
80	0.97179

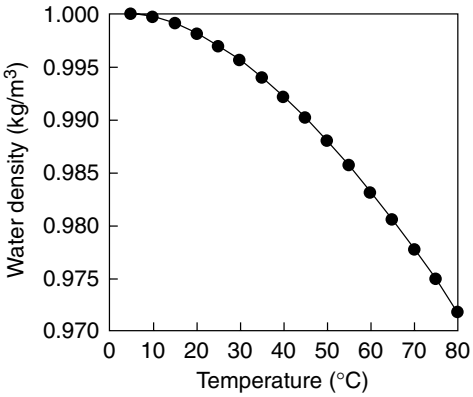

Figure D3.4.4 Density of pure water at 1 bar atmospheric pressure as a function of temperature.

6. Repeat with sample emulsion to determine its density (ρ).

   If necessary, the sample can be slightly warmed (see step 1).

7. Calculate the dispersed-phase volume fraction (ϕ) of the emulsion using the equation $\phi = (\rho - \rho_d)/(\rho_c - \rho_d)$.

BASIC PROTOCOL 2

MONITORING VISIBLE LAYER BOUNDARIES DURING STORAGE

The so-called creaming tests are a variation of the method described above (see Basic Protocol 1) and are mostly used for emulsions that destabilize in less than a week. These tests are particularly useful to determine the effect of critical parameters such as ionic strength, pH, or the addition of biopolymers or surfactants on the stability of emulsions. They are used as an experimental tool to gain insight into the causes of emulsion instability. These tests are not designed to obtain information about infinitesimal changes in particle size distribution and concentration during long-term storage of the emulsion.

Materials

Sample emulsion
Glass cylinder
Environmental chamber
Ruler
Strong light source

1. Fill glass cylinder with sample emulsion.

   Although there is no need for a certain size cylinder, the cylinder should have a sufficient height to clearly detect the visible boundary that forms as droplets rise to the top or sink to the bottom under the influence of the gravitational field. Throughout the experimental series, the type of glass cylinder used should be consistent.

2. Seal the cylinder and store in an environmental chamber.

   Increasing the temperature can accelerate the development of emulsion instability, thus reducing the required test time; however, raising the temperature causes a variety of other

changes. Mass transport processes are accelerated, droplet-droplet interactions are altered, and phase transitions in surfactant structures (i.e., micelles, bilayers, vesicles) may occur (Weiss, 1999). If stability of different emulsions is to be compared, it is important to conduct all experiments at the same temperature.

3. Remove the cylinder from the environmental chamber at regular intervals and measure the height of the visible boundary with a ruler. To increase contrast for better readings, use a strong light source to illuminate the emulsion background (i.e., provide backlighting).

   The precise determination of the location of the visible boundary layer is often difficult, particularly in the initial phase of the gravitational separation.

4. Plot boundary height as a function of storage time.

   For an example using this analysis, see Anticipated Results.

TEST ACCELERATION AND ULTRASONIC OR INFRARED SCANNING OF CONCENTRATION AND DROPLET-SIZE PROFILE

ALTERNATE PROTOCOL

Test acceleration

The most common method to increase emulsion destabilization kinetics is by utilizing a centrifuge to accelerate the gravitational separation of dispersed phase particles (Latreille and Paquin, 1990). By increasing or decreasing the number of revolutions per minute (rpm), and thus the strength of the centrifugal field, the kinetics of the emulsion breakdown can be changed. For systems with low viscosities of the aqueous phase, classical laboratory centrifuges that produce accelerations between 500 and $4000 \times g$ are usually sufficient. The utilization of ultracentrifuges with rotational accelerations of 15,000 to $50,000 \times g$ often results in a complete phase separation. While it may be useful for quantitative measurements of dispersed phase properties to completely break the emulsion, no information about the kinetics of the destabilization process is obtained. The speed of the centrifuge is therefore an experimental variable that may need to be adjusted depending on the nature of the emulsion. As previously mentioned, temperature-accelerated storage stability tests are not recommended due to changes in mass transport processes, phase transitions, and variations in droplet-droplet interactions, unless the elevated temperature accurately reflects the conditions encountered in the production, storage, and consumption setting.

Optical and ultrasonic monitoring of creaming profiles

The test procedures described above (see Basic Protocols 1 and 2) have recently been simplified by utilizing advanced techniques such as ultrasonic and infrared spectroscopy. The purchase of one of these advanced analytical instruments is recommended for emulsion manufacturers that frequently conduct emulsion stability tests and require automated analysis of a large number of samples.

Figure D3.4.5 illustrates the principle of the measurement. An example of a commercially available unit is the QuickSCAN Analyzer (Coulter-Beckman). These instruments allow the nondestructive recording of emulsion droplet size and concentration as a function of container coordinate x. They are designed around the concept of a reading head that combines transmission and backscattering of electromagnetic or ultrasonic waves. A motor moves the sample vial vertically relative to the reading head. In the case of the QuickSCAN Analyzer, the function of the reading head is based on the transmission and scattering of light in the near-infrared region (850 nm) where there is little absorbance of light. The system is able to accurately determine droplet concentrations of $<0.1\%$. At low droplet concentrations, the intensity of transmitted light is primarily analyzed, whereas

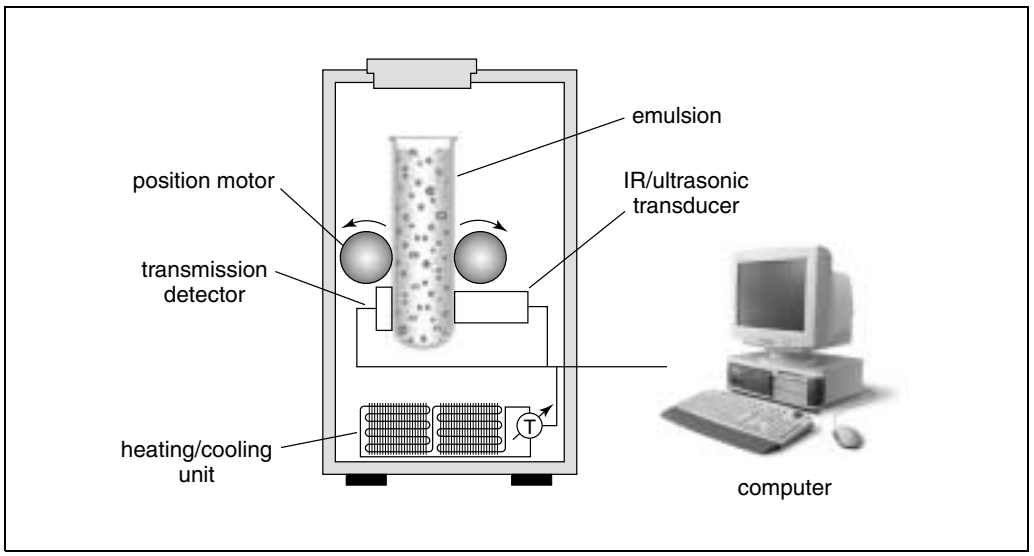

Figure D3.4.5 Schematic diagram of infrared/ultrasonic scanner to determine creaming profiles.

in the case of an optically opaque system, the intensity of backscattered light is evaluated. The advantage of a system based on both transmission and backscattering is that emulsion droplet concentrations can be measured over a wide range (0.1% to 50% w/v); however, the particle size distributions obtained from these measurements are not very accurate since the intensity of backscattered light is only recorded at a single scattering angle.

An alternative to the light scattering–based device has been developed by McClements at the University of Massachusetts (McClements, 1996; Basaran et al., 1998). The noncommercial experimental setup utilizes ultrasound to analyze the colloidal properties of the emulsion. Low-intensity high-frequency sound waves are emitted from an ultrasonic transducer and transmitted through the sample. A portion of the mechanical energy of the sound wave is absorbed by emulsion droplets and converted into thermal and mechanical energy, resulting in an attenuation of the wave. Changes in the velocity of the ultrasonic wave combined with the observed attenuation can be used to obtain precise information about the droplet size and concentration of emulsions. There are, however, a number of disadvantages to this technique. To receive a reasonably attenuated signal at the transducer, the emulsion droplet concentration should be >5% (v/v). In addition, a large number of dispersed- and continuous-phase physical properties are required for the subsequent determination of droplet size distribution. These physical properties include heat capacity, thermal expansion coefficient, and density. Their values may not be readily available in an industrial setting where material properties change on a daily basis. It should also be noted that the method is unsuitable for the characterization of foams.

Despite these limitations, both systems offer a considerable improvement over the previously introduced methods. They are considerably less labor intensive and thus may result in substantial cost reduction. Setup and operation of the units is simple, and both systems can be easily integrated into existing computer-driven quality assurance programs.

COMMENTARY

Background Information

Production of emulsions

The goal of food emulsion manufacturers is to produce emulsions that meet or exceed the expectations of their clientele. As a first step, companies typically conduct market studies to determine what these expectations are. Sensory evaluations are then used to translate these expectations into product-specific criteria (e.g., emulsion color, texture, appearance) that serve as guidelines to design the emulsification process and verify the quality of the produced emulsion. If emulsion properties comply with the set standards (i.e., their values are within an acceptable range), manufacturers can be confident that their customer base will be satisfied with the product.

At first sight, the above outlined product development scheme seems straightforward and fairly simple to implement. In reality, however, it can be a daring task. Two important skills need to be mastered. First, emulsion manufacturers must be able to actually *produce* emulsions that have the desired properties as specified by the previously mentioned product criteria. This requires that they are able to carefully control the outcome of homogenization, the process whereby large droplets are disrupted by mechanical forces to produce smaller ones (McClements, 1999). Homogenization is usually a two-stage process. In the first stage, two immiscible liquids are mixed in a high-speed blender to produce a coarse emulsion that consists of fairly large droplets (mean droplet sizes often exceed 10 μm). In the second step of the homogenization process, large droplets are further disrupted to produce droplets with small mean diameters (<1 μm; Weiss, 1999).

Once the emulsion has been produced, manufacturers must be able to guarantee that emulsion properties do not significantly change until the product is consumed—i.e., the emulsion has to remain *stable* after homogenization. While the first goal of producing a specific emulsion is already challenging as it requires an in-depth understanding of fluid dynamics and interfacial physics (Friberg and Karsson, 1997), it is the latter task (to produce stable emulsions) that poses the biggest problem (Weiss, 1999), because emulsions are inherently unstable (Hiemenz and Rajagalopan, 1997). To explain this, we have to briefly consider the fundamental nature of emulsions.

Nature of emulsions

Emulsions consist of two or more completely or partially immiscible liquids, such as oil and water, where one liquid is being dispersed in the other in the form of droplets (Dickinson, 1992). At the interface of each droplet, the molecules of the two liquids are in direct contact with each other, which is thermodynamically highly unfavorable. Although the entropy of the system increases as the emulsion is homogenized (increased entropy of mixing), this effect is not sufficient to balance the unfavorable enthalpy increase that arises because of the contact between hydrophilic and hydrophobic molecules (Hiemenz and Rajagalopan, 1997). The system has the tendency to phase separate to attain a configuration in which the contact area between the two phases, and subsequently the free energy of the system, is minimal. Surfactant molecules that adsorb at liquid-liquid interfaces can decrease the enthalpy contribution to the overall free energy increase and thus reduce the tendency of the emulsion to destabilize (Weiss, 1999). However, although surfactant molecules can reduce the tendency of an emulsion to destabilize, they are not able to completely prevent it. From a thermodynamic perspective, emulsion destabilization is inevitable. Fortunately, emulsion breakdown does not occur instantaneously. A finite time is required for droplets to collide, merge, coalesce or grow, and eventually phase separate. If this process occurs over a long period of time that exceeds the intended lifetime of the product, the emulsion is considered to be *kinetically* stable (Weiss, 1999).

In the broadest sense, stability of emulsions should be defined as maintenance of an initial state that was attained after homogenization of the two (or more) liquids (Sjöblom, 1996). The initial state of the emulsion can be defined by a set of internal parameters. The primary parameters used to describe the state of an emulsion are droplet size distribution and concentration, since the bulk properties of emulsions such as color, texture, and taste are primarily a function of these two colloidal parameters (McClements, 1999). In selected cases it may be necessary to include additional parameters such as pH and microbial load to further define the initial state of the emulsion.

The lifetime of emulsions is a function of various extrinsic parameters. During production, transport, and storage, emulsions are subject to a variety of fluctuating external stresses

that can alter the initial state of the system and eventually cause the emulsion to become unstable (Figure D3.4.1). The goal of any emulsion stability test should be to closely model stress conditions encountered in an actual application setting. Most emulsion stability tests focus on the measurement of droplet size distribution and concentrations as a function of storage time at a specific temperature. Figure D3.4.2 illustrates that emulsions may be subject to a variety of chemical, physical, and microbiological stresses in addition to temperature. All these stresses can have profound effects on the macroscopic and colloidal properties of emulsions (Sjöblom, 1996). It should be noted that measurements of bulk properties such as rheology and color as a function of time can be used to determine the stability of emulsions as well (Serra and Casamitjana, 1998); however, interpreting bulk emulsion properties to obtain information about changes on the colloidal level is much more difficult than measuring colloidal properties to predict changes on the macroscopic level.

Instability mechanisms

The following sections discuss the various instability mechanisms that result in the breakdown of emulsions. Because most of these instability mechanisms are driven by droplet-droplet interactions that occur on the colloidal level, the physical bases of colloidal interactions should be understood as well. Such a detailed discussion is, however, beyond the scope of this unit and interested readers are referred to the comprehensive reviews of Dickinson (1992), Israelachvili (1992), and McClements (1999).

Emulsions are thermodynamically unstable systems because of the unfavorable interactions between polar and nonpolar phases. These systems have the tendency to minimize the contact area between the two opposing phases by merging smaller droplets into larger ones. Eventually, the phases will separate, causing the emulsion to be "broken" (Dickinson, 1992). Emulsion instability can be caused by molecular as well as supramolecular mass transport mechanisms (Weiss, 1999). Figure D3.4.6 shows an overview of emulsion instability mechanisms. Supramolecular mechanisms are flocculation, creaming or sedimentation, partial or complete coalescence, and phase inversion. Ostwald and compositional ripening are examples of instability phenomena that are based on the transport of single dispersed phase molecules through the intervening aqueous phase by means of molecular diffusion (Weiss, 1999). It has been increasingly recognized that these molecular mass transport mechanisms can dominate the overall instability of the emulsion, and research efforts have recently focused on these mechanisms. It is generally important to be able to recognize which mechanism is responsible for emulsion breakdown, since the methods used to counteract each of the instability mechanisms differ greatly.

1. *Creaming or sedimentation*. Creaming or sedimentation is one of the principal instability mechanisms seen in emulsions. Emulsion

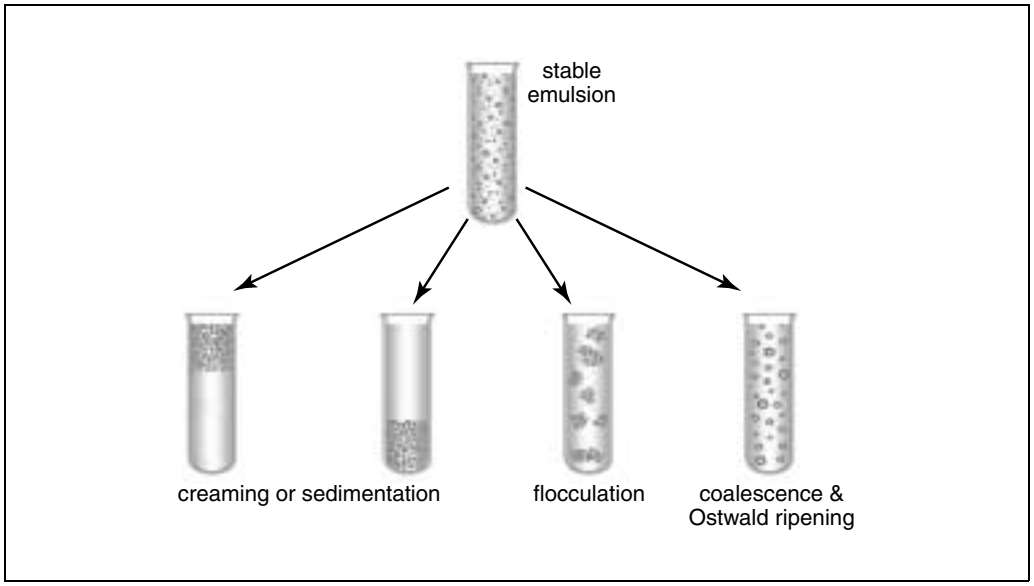

Figure D3.4.6 Comprehensive overview of the principal mechanisms that cause emulsion instability.

droplets cream or sediment if a density difference exists between the continuous and dispersed phases. A force balance done on a single droplet with a given size results in the well-known Stokes law, which describes the velocity of the upward or downward motion of a droplet as a function of droplet radius (Dickinson, 1992). A single, isolated, rigid, uncharged droplet with radius r and density ρ, suspended in a continuous phase of density ρ_0, experiences a buoyancy force F_b in the opposite direction of a gravity field with acceleration g that can be expressed as:

$$F_b = \frac{4}{3}\pi r^3 g(\rho_0 - \rho)$$

The droplet simultaneously experiences a frictional force due to the dynamics of the surrounding fluid that opposes its movement. Under laminar flow conditions, the frictional force is given by $F_f = 6\pi\eta_0 rv$, where η_0 is the shear viscosity of the medium and v the velocity with which the droplet moves. Under steady-state conditions, the so-called Stokes velocity (v) emerges from the force balance:

$$v = \frac{2gr^2(\rho_0 - \rho)}{9\eta_0}$$

The Stokes velocity equation illustrates a number of important parameters. The frictional force that arises because of the movement of the droplet depends on the viscosity of the continuous phase. Increasing the viscosity of the external phase or decreasing the size of the droplets can therefore enhance the stability of emulsions. Also, decreasing the size of the droplets or the density difference between the two phases causes a decrease in the Stokes velocity. The Stokes equation therefore offers practical advice on how to improve the stability of emulsions. The use of the Stokes equation is, however, limited. Real emulsions are far from being monodisperse; in fact, they are substantially polydisperse. Real emulsions are also rarely infinitely dilute so that each of the droplets behaves like a single entity. Droplet-droplet interactions often result in the formation of complex three-dimensional floc structures that have increased hydrodynamic radii. Furthermore, the rheological behavior of the external phase may deviate from that of a Newtonian liquid, thus adding complexity to the flow situation. In conclusion, factors that limit the use of the Stokes equation to predict creaming or sedimentation behavior are high disperse volume fractions, flocculation phenomena, electrical charge of droplets, partial crystallization of dispersed phase molecules, and the presence of an adsorbed layer (McClements, 1999). These factors have to be considered in the interpretation of the Stokes equation.

2. *Flocculation*. Flocculation is the process whereby two or more droplets come together and form an aggregate without losing their individual integrity (McClements, 2000). Flocculation depends on the frequency with which droplets collide and the efficiency of the collisions. Whether or not the droplets will actually aggregate after a collision is a function of the colloidal interactions between the droplets (Sjöblom, 1996). In order for two droplets to become flocculated, the energy potential of the droplet pair (the interdroplet potential) must be negative at some separation distance. At this separation distance, the system has the most stable configuration (that is, its free energy is minimal). How strongly the droplets are bound to each other depends on the depth of the potential well.

Attractive interactions are a function of a variety of parameters such as temperature, ionic strength, and charge of the interfacial layer (Israelachvili, 1992). Droplet-droplet interactions can be increased if substances are added to the aqueous phase that cause bridging between the two droplets. An example would be the presence of multivalent ions such as calcium or magnesium. Other factors that may increase the attraction between two droplets include the hydrophobicity of droplet surfaces (due to insufficient coverage of the droplet interface with surfactant molecules) or depletion interactions (due to the addition of large polymers to the surrounding aqueous phase). Repulsive interactions, on the other hand, may be reduced if interfacial properties, such as layer thickness or interfacial layer charge density, are altered (Israelachvili, 1992). The strength of the interaction will ultimately influence the structure of flocs. In weakly flocculated emulsions with weak attractive forces between the droplets, the flocs tend to have a more globular structure, whereas in strongly flocculated emulsions, an elongated network of droplets can be formed. Since the speed of creaming depends on the effective particle size (see above), flocculated emulsions will be more prone to creaming than nonflocculated ones. An additional factor to consider is that the formation and structure of flocculated aggregates also alters the rheological properties of emulsions (Serra and Casamitjana, 1998). An

emulsion that contains aggregates shows a more pronounced shear-thinning behavior than nonflocculated emulsions, because flocs are disrupted as a mechanical shear stress is applied. Thus, at higher shear rates, the emulsion will behave closely to a nonflocculated emulsion where droplets do not interact with each other.

3. *Coalescence*. If two droplets come into close contact for an extended period of time (e.g., due to flocculation or accumulation in a creamed layer), the small liquid film that separates the two droplet membranes will gradually start to thin (Dickinson, 1992). The speed with which this film thinning occurs is a function of the hydrodynamic properties of the film, the colloidal interactions between the two membranes, and the membrane composition itself. The thickness of the film can vary with time. The close contact of the two membranes will eventually cause them to become distorted. At this point, a thin liquid lamella can be formed between the two droplets, with the liquid gradually draining from the film. The persistent fluctuations in the interfacial membranes due to the high mobility of molecules at the interface thus cause the rupture of the film and the two droplets will spontaneously merge. This process is irreversible unless the emulsion is homogenized again. Coalescence dramatically differs from flocculation in that flocculation may be reversed by the application of low shear forces (Sjöblom, 1996). Coalescence depends strongly on the interfacial properties of the system (McClements, 1999). Proteins are in general ideally suited to stabilize emulsion droplets and foams against film rupture. The interfacial membrane formed by proteins is highly viscoelastic and therefore dampens the modulations that occur at the interface extremely well. It should be mentioned that this beneficial effect is often counteracted by the slow adsorption kinetics of proteins, which negatively impacts homogenization efficiency. If the interface between oil and water cannot be completely covered during homogenization, the droplets will immediately recoalesce. Hence, it is more difficult to produce small emulsion droplets that are stabilized by proteins.

4. *Partial coalescence and phase inversion*. Partial coalescence and phase inversion are additional mechanisms that can destabilize an emulsion (Dickinson, 1992; Friberg and Karsson, 1997; McClements, 1999). Partial coalescence occurs when emulsion droplets are cooled below the melting point of the dispersed phase (Rousseau, 2000). As a result, the molecules of the dispersed phase may undergo a phase transition and spontaneously crystallize. The growing crystals can form extremities that extend beyond the formerly smooth interfacial layer of the dispersed particles, thus disrupting the protective interfacial layer. The presence of these crystalline extremities causes the particles to rapidly aggregate upon collision. The crystal from one droplet may penetrate the interface of another droplet causing them to stick together (Rousseau, 2000).

Another form of instability refers to changes in the emulsion type—i.e., the emulsion may spontaneously revert from an oil-in-water to a water-in-oil emulsion or vice versa. This process is called phase inversion (Dickinson, 1992). Phase inversion occurs primarily in emulsions that have very high volume fractions. This process is closely associated with the properties of the emulsifier. An example would be the addition of multivalent cations to a highly concentrated oil-in-water emulsion stabilized by a cholesterol–sodium cetyl sulfate film. The interfacial layer of this particular emulsion consists of a combination of cholesterol–sodium cetyl sulfate and is negatively charged. As multivalent cations are added, the surface charge is neutralized, thereby causing the droplets to aggregate. The coagulating oil droplets entrap pockets of water; however, because of the nature of the surfactant layer, the layer does not thin and disrupt, which would result in immediate coalescence. Instead, it realigns in such a way that the water droplets are now stabilized within the surrounding oil phase, so that a water-in-oil emulsion is formed. It is fairly easy to detect phase inversion, as the process generally results in sudden alterations in emulsion conductivity and viscosity (Latreille and Paquin, 1990).

5. *Ostwald ripening.* Ostwald ripening or transcondensational ripening is the process whereby larger droplets grow at the expense of smaller ones, because of the transport of dispersed phase molecules from smaller to larger droplets through the intervening continuous phase (Weiss, 1999). The driving force for this process is an increase in solubility of dispersed phase molecules in the continuous phase, which occurs when the droplet curvature increases (i.e., the droplet size decreases). This can be seen in the expression:

$$c_r = c_{r,\infty} \exp\left(\frac{2\gamma V_m}{rRT}\right)$$

where c_r is the solubility of the dispersed phase, r the curvature of the interface, $c_{r,\infty}$ the solubility of the dispersed phase having a planar interface, γ the interfacial tension, R the universal gas constant, T the temperature of the system, and V_m the molar volume of the dispersed phase.

In a polydisperse system, the number of dissolved dispersed phase molecules that surround small droplets is higher than the number of molecules that surrounds large droplets. As a result of this concentration gradient, there is a net movement of molecules from smaller to larger droplets. Smaller drops are said to "dissolve" and diffuse through the aqueous phase to "recondense" onto larger droplets. The theory that describes the process of Ostwald ripening was initially formulated by Wagner (1961) and independently by Lifshitz and Slyzov (1961). Assuming that the particle concentration in the system is small and that mass transport of dispersed phase molecules is limited to diffusion, the rate of Ostwald ripening (ω; i.e., the time-dependent increase of the mean droplet size, $\overline{r}$) is given by:

$$\overline{r}(t) = \left(\frac{8\gamma D c_{r,\infty} V_m^2 t}{9RT} \right)^{\frac{1}{3}}$$

where D is the diffusion coefficient, V_m the molar volume, $c_{r,\infty}$ the solubility of dispersed phase molecules, γ the interfacial tension, and T the temperature of the system. Ostwald ripening is primarily influenced by the solubility of the dispersed phase molecules in the aqueous phase. The lower the molecular weight of dispersed phase molecules, the higher the solubility in the aqueous phase. The presence of substance that increases the solubility (e.g., surfactant micelles) can strongly accelerate emulsion destabilization. In recent studies, it has been shown that an increase in the number of surfactant micelles can increase the rate of Ostwald ripening (Weiss, 1999). This was explained in terms of the surfactant micelles acting as a carrier of dispersed phase molecules between droplets. This is an important factor to consider. Often emulsion manufacturers react to a situation in which their emulsions rapidly destabilize by simply adding more surfactants. If the destabilization is caused by incompletely covered droplet interfaces that are prone to coalescence, this method can indeed increase emulsion stability. If, however, the emulsion is destabilized by Ostwald ripening, then the addition of more surfactant would actually accelerate the emulsion breakdown.

Recommendations to increase emulsion stability

The determination of emulsion stability is of considerable importance. In order to effectively increase the stability of emulsions, emulsion manufacturers must be able to determine the reason for the apparent instability (i.e., to identify the associated instability mechanism). Coalescence is often caused by an incomplete coverage of droplets with surfactant molecules. An increase in the surfactant concentration or a replacement of the emulsifier being used can often improve the resistance of emulsions to coalescence. Since coalescence is a function of droplet collisions with a subsequent merger of interfacial membranes, layers with strong repulsive interactions are better suited to stabilize emulsion droplets. A strong repulsive force may prevent collisions between droplets in the first place. Repulsive interactions can be altered by modifying the charge at the droplet surface or by using a surfactant that has a different thickness of the interfacial layer. If emulsion droplets become flocculated, attention should be focused on droplet-droplet interactions. Flocculation is often caused by the presence of small concentrations of biopolymers in the aqueous phase that cause depletion flocculation. The remedy is to either increase or decrease the amount of biopolymer in the system, since flocculation tends to occur over a narrow range of polymer concentration. The identification of Ostwald ripening as the major instability mechanism has important consequences. In this case, the addition of surfactant molecules will not reduce the kinetics of emulsion breakdown. On the contrary, it may actually accelerate the breakdown process (Weiss, 1999). Since Ostwald ripening is a function of the solubility of the dispersed phase, the addition of a second nonpolar lipid with a substantially lower solubility to the high-solubility dispersed phase can strongly reduce the growth of droplets.

Critical Parameters and Troubleshooting

Anticipated problems and counter measures for the listed protocols have been included in individual steps of the basic protocols. In addition, a detailed troubleshooting section for particle size analysis can be found in *UNIT D3.3*; however, the difficulty of properly conducting accelerated tests should again be stressed. While the method is extremely useful, as it can drastically reduce the required time for the overall test, results may not accurately reflect

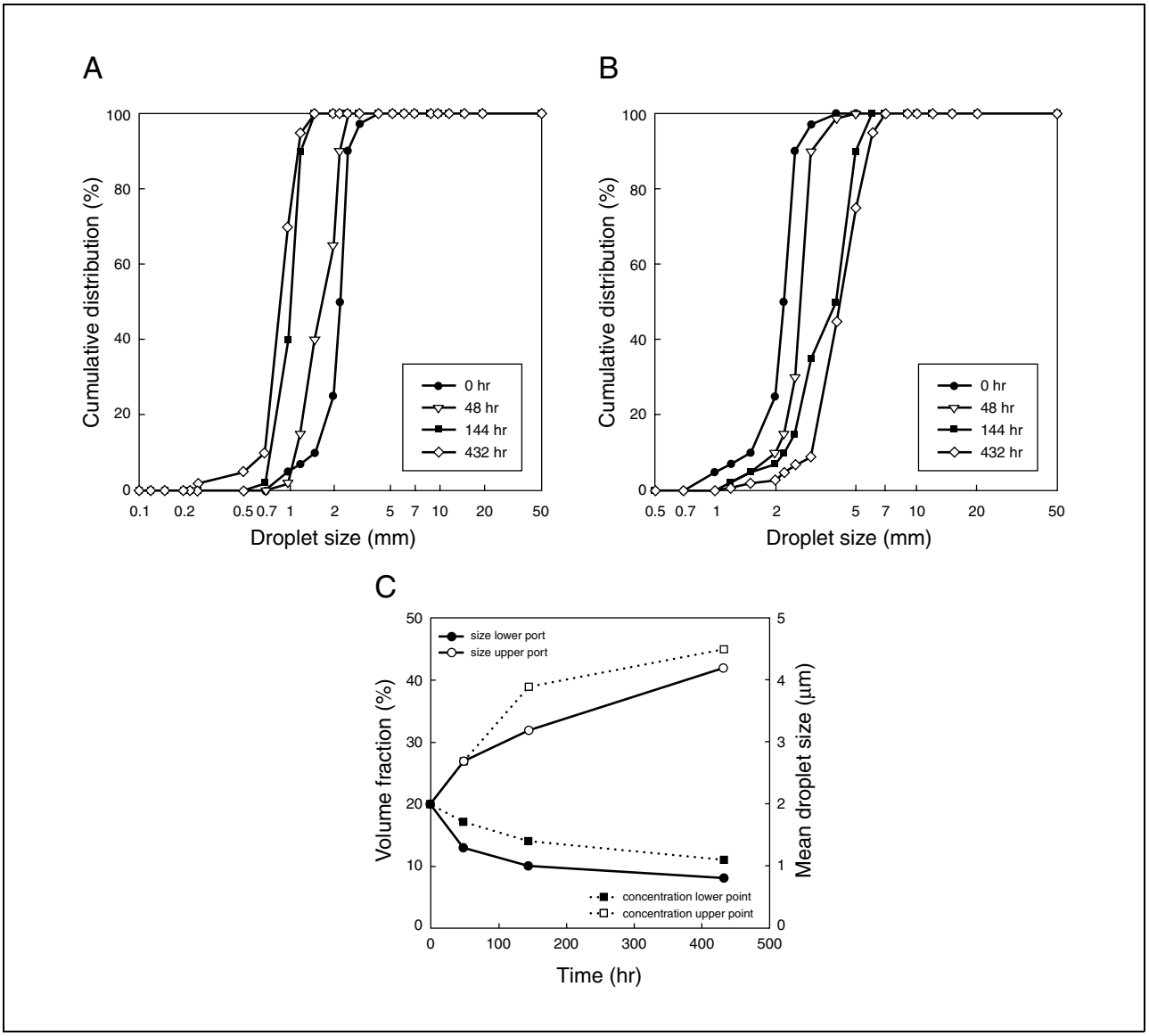

Figure D3.4.7 Change in cumulative particle size distribution of a 20% (w/v) oil-in-water emulsion stabilized by 2% (w/v) Tween 20 at the lower port (**A**) and upper port (**B**). (**C**) Change in mean droplet diameter and volume fraction of the emulsions as a function of time.

the stability of the product during storage on a supermarket shelf. If the gravitational acceleration is too high during acceleration of the test, interfacial membranes may be subjected to stresses that are not representative of normal storage conditions and the system can exhibit rapid coalescence.

Anticipated Results

Storage stability test

Figure D3.4.7 shows typical results obtained from a storage stability test (see Basic Protocol 1) of an oil-in-water emulsion that consists of a 20% (v/v) hexadecane-in-water emulsion stabilized by 2% (w/v) polyoxyethylene-20-sorbitan monolaureate (Tween 20). The emulsion was stored at 30°C for up to 500 hr. By this time, the emulsion underwent extensive creaming and coalescence. Consequently, the cumulative size distribution of emulsion samples withdrawn from the lower port (Figure D3.4.7A) decreases as time progresses, while the droplet size distribution of samples withdrawn from the upper port simultaneously increases (Figure D3.4.7B). A plot of the cumulative size distribution is better suited than a plot of the differential (or frequency) size distribution to compare a large number of droplet populations. The cumulative size distribution at a particular droplet size (r_p) is simply the sum (or integral) of the differential size distributions

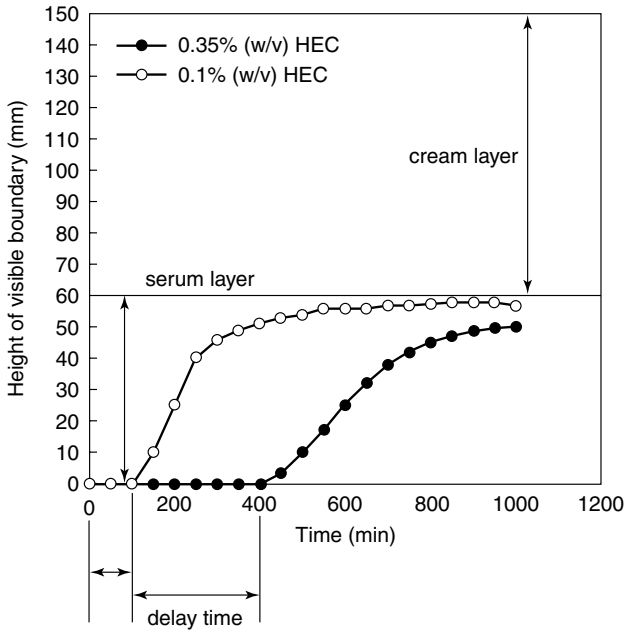

Figure D3.4.8 Influence of polymer concentration on the time-dependent height increase of the visible creaming layer. Adapted from Manoj et al. (1998) with permission from Academic Press.

from r_{min} to r_p. The software of most modern particle size analyzers offers the option to convert measured frequency size distributions into cumulative size distributions. An algorithm to convert differential size distributions into cumulative size distribution can be found in Weiss (1999).

A summary of the storage stability test is shown in Figure D3.4.7C, which shows a plot of the mean droplet size and concentration as a function of storage time. The graph illustrates that both the mean droplet size and the volume fraction of the emulsion increase at the upper port and decrease at the lower port as the emulsion ages. The spatial distributions of both droplet size distribution and concentration change profoundly as the emulsion destabilizes. An interesting result of the stability test is that the mean droplet size increases overproportionally at the upper sampling port when compared with mean droplet size changes observed at the lower port. This can be explained in terms of frequency of droplet collisions (Robins, 2000). Since the droplet concentration at the upper port continuously increases, the probability for droplet-droplet collisions resulting in coalescence is increased.

This example demonstrates that storage stability tests are extremely useful as they allow emulsion manufacturers to accurately follow even small changes in emulsion properties. Plots of droplet size distribution and concentration as a function of time can be used to determine the kinetics of the instability process and to determine the shelf life of the product by setting upper and lower limits for both mean droplet size and concentration at each port.

Creaming test

Despite the simplicity of the procedure, creaming tests (see Basic Protocol 2) are extremely useful to study instability mechanisms. Figure D3.4.8 shows an example of results obtained from a creaming test adopted from Manoj et al. (1998). This experiment evaluated the effect of 0.1% and 0.35% (w/v) hydroxyethylcellulose (HEC), a high-molecular-weight biopolymer, on the stability of *n*-hexadecane oil-in-water emulsions stabilized by 34% (w/v) polyoxyethylene 23-lauryl ether (Brij 35) at 25°C. As can be seen from Figure D3.4.8, the height of the visible boundary between the serum and the cream layer increases as the emulsion ages, indicating that a compact cream layer is formed. At higher concentrations

of HEC, the height of the visible boundary layer increases less rapidly than at lower concentrations. For example, the height of the visible boundary of an emulsion containing 0.1% HEC is 55 mm at 500 min, as opposed to 10 mm for an emulsion containing 0.35% HEC.

These results are typical of emulsions that become depletion flocculated at low polymer concentrations and polymer stabilized at high polymer concentrations. Depletion flocculation is the result of an inhomogeneous distribution of polymer molecules throughout the colloidal system (Chanamai and McClements, 2001). The HEC concentration in the vicinity of oil droplets is lower than the concentration of HEC in the bulk phase because of the finite size of the cellulose molecules. Since HEC is a nonadsorbing polymer, the concentration of HEC at the interface is zero. Consequently, an osmotic pressure gradient exists. The pressure gradient causes emulsion droplets to become aggregated in order to minimize the total volume that is void of HEC molecules. The flocculated droplets in turn have a larger apparent size and cream at a faster rate in the gravitational field. At high HEC concentrations, the viscosity of the continuous phase has been increased to a point where the movement of droplets is retarded. As a result, the emulsion creams at a much slower rate (McClements, 2000).

This example illustrates two important facts. First, the long-term stability of emulsions is strongly influenced by the addition of high-molecular-weight biopolymers. Adding low concentrations of hydrocolloids to emulsions can decrease the stability of emulsions, whereas the addition of a large concentration of biopolymer can contribute to enhancing the stability of emulsions (McClements, 2000). Second, it illustrates the difficulty involved in producing stable emulsions. An in-depth understanding of the colloidal nature of the system is ultimately required. Creaming tests provide a valuable tool to rapidly obtain information on how various parameters such as pH, ionic strength, or biopolymer concentration impact emulsion stability.

Time Considerations

Basic Protocol 1: Storage stability test

While the actual preparation time of samples is short and involves only several hours, the actual storage stability tests are lengthy and may be conducted over a period of more than a year. The total length of the experiment ultimately depends on the shelf life stability requirements of the final product and thus will vary. Upon sample withdrawal, the analysis requires less than 30 min if the required methods (droplet size and concentration measurements) have been properly set up.

Basic Protocol 2: Creaming profile determination

As in the storage stability test (see Basic Protocol 1), the preparation time is short. Less than 30 min are required to set up the samples for storage. The actual storage stability for creaming profile determination is usually conducted over a periods of up to 4 weeks. Tests that are accelerated by centrifugation may require less than 8 hr of preparation and completion.

Support Protocol: Density measurement

The required time depends strongly on the equipment being used. Modern analytical equipment can correctly measure density within minutes. If the density measurement is conducted using the specific gravity bottle method, then an initial calibration is required that can take up to several hours (due to the fact that the volume of the bottle has to be determined as a function of temperature). After the initial calibration curve has been obtained, tests can be conducted within 15 to 20 min since only a precise mass determination is necessary.

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Israelachvili, 1992. See above.

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McClements, 1999. See above.

Detailed description of theory, measurements, and applications in emulsion science covering all aspects of emulsion stability.

Weiss, 1999. See above.

Thorough investigation of emulsion instability caused by molecular mass transport processes.

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Key Concepts of Interfacial Properties in Food Chemistry

The majority of foods exist as highly dispersed systems, that is, they consist of multiple phases (solid, liquid, or gas; Table D3.5.1). These typically multicomponent phases are separated by a narrow region, the phase boundary or interface/surface, in which the molecules of the nonimmiscible phases may be in direct contact with each other. Many important chemical and physical processes are influenced by the presence of an interfacial region. Due to the existence of an interfacial layer, active components such as free radicals or antioxidants can partition between the different phases and reduce or increase the concentration of active components in the reactant phase. Partitioning may consequently increase or decrease the rate with which the chemical reaction proceeds. In addition, the nature of the interface itself may directly affect the reaction. A variety of natural substances are surface active, that is, they adsorb readily at interfaces and form so-called interfacial membranes. These interfacial membranes compartmentalize the phases and prevent the molecules of the different phases from being in direct contact with each other. If the mechanism of chemical reaction requires direct interaction of the two species (i.e., no intermediates are formed), then the rate of the chemical reaction can be decelerated. Depending on the nature of the interfacial membrane, it is also possible to completely prevent mass transport of a reactive species across phase boundaries. This principle has in fact been utilized in nature for millions of years and is the foundation of all life on this planet. Cell membranes typically carry a charge that prevents similarly charged molecules from diffusing through the interfacial membrane. The electrostatic repulsive interactions that are the basis of this phenomenon help to create an environment in the interior of the cell that is rich in ions. The controlled release of these ions through appropriate ion channels is then utilized by the cell to produce ATP, the energy-rich compound that drives many biochemical reactions. This important example illustrates that interfacial properties are of utmost importance in chemical, biological, and physical processes. Since all these processes play a role in food systems, it is apparent that food chemists need to be concerned about interfacial properties of foods as well (Kinsella and Whitehead, 1989; Krisdhasima et al., 1993; Chen and Dickinson, 1995a-c; Dalgleish, 1996; Lin and Timasheff, 1996; Malmsten, 1998; Dickinson, 1999, 2001; Rodriguez Patino and Rodriguez Nino, 1999; Rodriguez Nino et al., 1999; Holt, 2000; Rodriguez Patino and Dominguez, 2000; Wilde, 2000).

The role of interfacial properties in food science and technology has increasingly gained the attention of many researchers. Our enhanced understanding of interfaces and the nature of dispersed systems has, for example, resulted in the production of foods that are substantially more stable and have specific properties that meet required predesigned specifications. The purpose of this discussion unit is to familiarize food scientists with

Table D3.5.1 The Classification of Dispersed Systems is Based on the Nature of the Involved Phases

Continuous phase	Dispersed phase	Dispersed system
Gas	Liquid	Fog
Gas	Solid	Smoke
Liquid	Liquid	Emulsion
Liquid	Solid	Suspension
Liquid	Gas	Foam

Contributed by Jochen Weiss

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key concepts in interface and surface chemistry. First, the basic concepts of physical chemistry of interfaces are introduced. The primary interfacial properties that are of interest to food chemists are then derived and described. Finally, the mechanisms of lipid oxidation in emulsions, which is a good case study to highlight the role of these interfacial properties in a technologically important process such as lipid oxidation, will be discussed. For additional discussion on the specific case of emulsions, see *UNIT D3.4*.

A BRIEF INTRODUCTION INTO THE PHYSICAL CHEMISTRY OF INTERFACES

A better understanding of phenomena in surface or interfacial science unfortunately requires at least a rudimentary knowledge of the relevant fundamental thermodynamic concepts. Interfaces do not consist of a three-dimensional bulk phase, but rather a two-dimensional virtual plane that in many cases is not accessible to direct quantification techniques. The expressions that are derived in the following section are therefore required to obtain the properties of interest. In addition, they help generate an understanding and appreciation of the peculiar nature of interfaces. Even though the mathematical derivations may seem a bit tedious at first, they reveal the true strength of fundamental thermodynamics, which is to take an originally abstract concept and develop it into a directly applicable mathematical model that can be used to extract the parameter of interest from experimentally obtained results.

Interfaces, Surfaces, and the Gibbs Dividing Plane

What is an interface? To answer this essential question, first consider the molecular nature of the phases involved. Assume that the system in question has a simple structure and contains only two components, A and B. The molecular interaction between components A and B will ultimately determine the overall structure of the system. Three particular cases can be identified:

1. The interaction between components A and B (i.e., A↔B) is energetically more favorable than any A↔A and B↔B interaction. In this case, the system is miscible. The mixing process will lower the overall free energy of the system and thus stabilize the system.

2. The interaction between components A and B is energetically unfavorable compared to the other two molecular interactions. In this case, the system is immiscible. The phases will separate immediately upon mixing to minimize contact between the two species.

3. In some rare cases, there is no clear preference between the three types of interactions. In this case the system is said to be partially miscible. A broad interfacial region will be

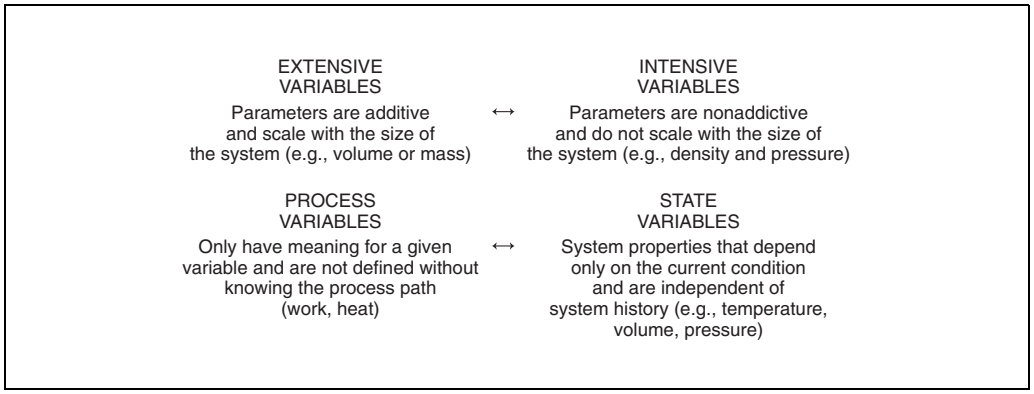

Figure D3.5.1 Basic macroscopic parameters of general thermodynamic systems.

formed as a result—e.g., water-acetone-methyl isobutyl ketone (a three component system) or short-chain alcohols and water (e.g., butanol-water).

Based on these three cases the most general definition of an interface can now be formulated. *An interface or surface is a narrow region that separates two completely or partially immiscible phases from each other.*

In order to be able to derive a mathematical description for the above-defined interface, a few basic thermodynamic principles have to be remembered. Thermodynamic systems are generally characterized by a set of independent macroscopic parameters that can be used to describe the state of a given system. These thermodynamic variables can be either state or process variables, or can be intensive or extensive variables. Figure D3.5.1 illustrates this fundamental thermodynamic concept. It is now time to reconsider the simple example in which two materials (phase α and β) are in direct contact. In this system, the inner energy of the interface can be designated as U^{σ}. The total energy of the system (U) is consequently the sum of the contribution of the inner energy of phase α (U^{α}), phase β (U^{β}), and the interface. Thus, one derives the following expression for the total inner energy of the system:

$$U = U^{\alpha} + U^{\beta} + U^{\sigma}$$

Equation D3.5.1

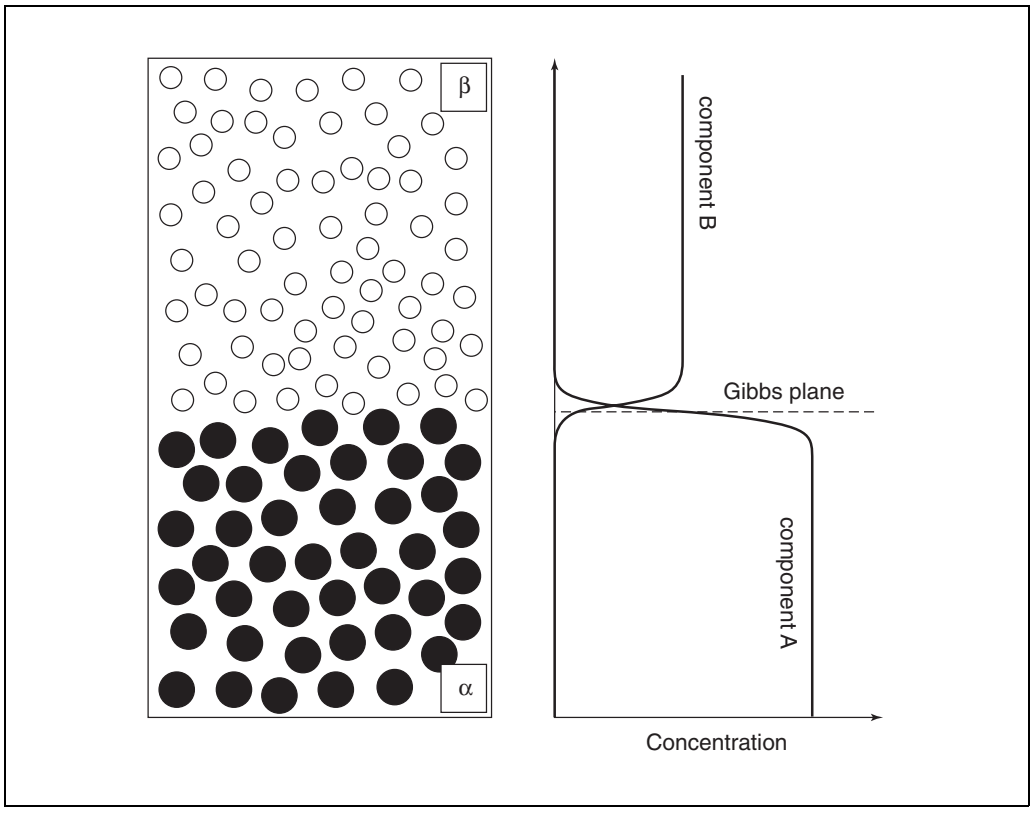

Figure D3.5.2 Definition of the Gibbs dividing plane based on the excess concentration of component A of the two phases α and β that are in direct contact with each other.

Replacing the inner energy terms with measurable quantities such as the volume-specific inner energies (U_V) will transform Equation D3.5.1 into:

$$U = V^{\alpha}U_V^{\alpha} + V^{\beta}U_V^{\beta} + U^{\sigma}$$

Equation D3.5.2

Equation D3.5.2 clearly illustrates that in order to derive an expression for the state of the interface, it is necessary to know the absolute volume of each of the phases; however, questions arise. How is the volume of each phase defined? Where does one phase end and another begin? To solve this dilemma, the so-called Gibbs convention was introduced. Gibbs concluded that if it was not possible to exactly determine how the concentration of molecules A and B are distributed across the interface, then a mathematically thin plane would have to be designated in which the *excess* of one component would by definition be zero. This mathematical plane is referred to as the Gibbs dividing plane. Figure D3.5.2 shows an example of a Gibbs dividing plane that was defined using phase α as the reference. The excess of molecular species A (n_α) at the interface (σ) is zero by definition:

$$n_{\alpha}^{\sigma} = 0$$

Equation D3.5.3

This definition can be conveniently used to introduce the term of greatest interest, the so-called interfacial coverage (Γ):

$$\Gamma_{\alpha} = \frac{n_{\alpha}^{\sigma}}{A}$$

Equation D3.5.4

where A is the interfacial area. As discussed later (see Interfacial Properties), the interfacial coverage is a very important quantity. It allows the determination of the specific amount of a particular component that may be present within the interface. This quantity is extremely useful if there is an accumulation of a third component at the interface, thus indicating whether this third component is surface active.

Interfacial Tension: A Key Parameter to Describe Interfacial Phenomena

Interfacial tension is one of the key parameters to determine the properties of interfaces, because (1) it is one of the few parameters than can be directly measured and (2) it can be measured by a large variety of experimental methods. The particular usefulness of surface tension comes from the fact that it may be used to gain information about other interfacial properties such as surface coverage (see Interfacial Properties).

The thermodynamic derivation of the interfacial tension is a fairly straightforward process. In a multiphase system in which the composition is not altered, the overall free energy (G) is a function of temperature (T), pressure (P), and interfacial area (A):

$$G = f(P,T,A)$$

Equation D3.5.5

The state and stability of the system is hence altered if one of the system variables is varied. The subsequent change in the free energy (dG) can then be calculated from the sum of the partial derivatives of the free energy:

$$dG = \left[\left(\frac{\partial G}{\partial T}\right)_{A,P=const} dT\right] + \left[\left(\frac{\partial G}{\partial P}\right)_{A,T=const} dP\right] + \left[\left(\frac{\partial G}{\partial A}\right)_{P,T=const} dA\right]$$

Equation D3.5.6

Under the assumption that the pressure and temperature of the system remain constant (i.e., dT and $dP = 0$), Equation D3.5.6 simply states that:

$$dG = \left(\frac{\partial G}{\partial A}\right)_{P,T=const} dA$$

Equation D3.5.7

The partial derivative of the Gibbs free energy per unit area at constant temperature and pressure is defined as the interfacial coefficient of the free energy or the interfacial tension (γ), a key concept in surface and interface science:

$$\gamma = \sigma = \left(\frac{\partial G}{\partial A}\right)_{P,T=const}$$

Equation D3.5.8

γ and σ are in fact interchangeable symbols even though it should be noted that γ is the preferred symbol for the interfacial tension and σ the preferred symbol for the surface tension. One commonly refers to surfaces if one of the phases is a gas. In a broader sense, however, a surface may simply be defined as a special case of an interface. Hence, this chapter primarily refers to interfaces. Equation D3.5.8 also offers a simple explanation for the fact that dispersed systems such as emulsions have the tendency to minimize surface or interfacial areas between phases. If the interfacial area is expanded, the overall free energy of the system will increase. This increase in total energy of the system also

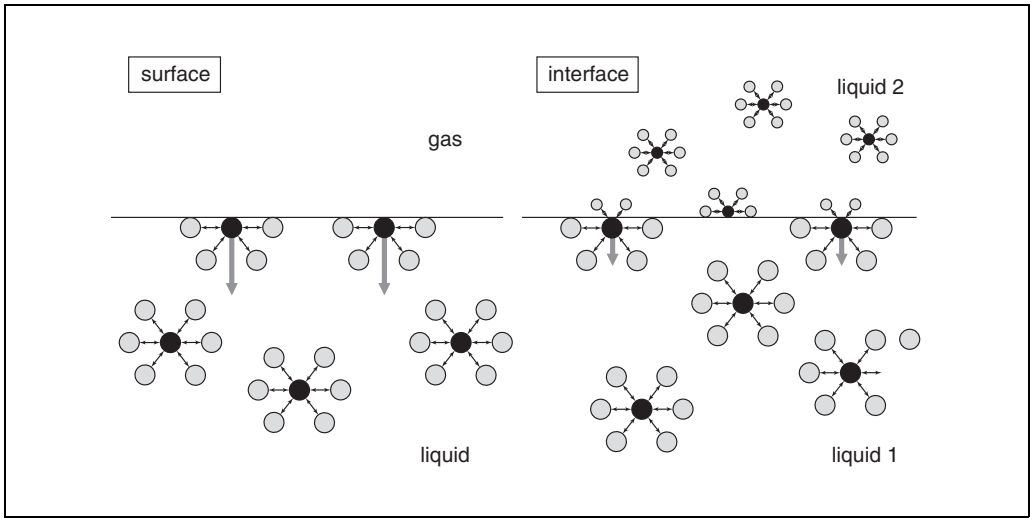

Figure D3.5.3 The molecular interactions of molecules in adjacent phases can be used to visualize the concept of surface or interfacial tension.

explains why work (W) is required to form new interfaces. For example, a large amount of mechanical energy is usually required to homogenize emulsions:

$$W = \gamma \Delta A$$

Equation D3.5.9

The interfacial tension appears to an external observer as a virtual force. To visualize the concept of this virtual force, reconsider the interactions between the two molecular species that are present in neighboring phases. Molecules that are located within the interface will have a weaker interaction with molecules in the neighboring phase than with molecules in their own bulk phase. The result is a directional force that is perpendicular to the interface and directed into the bulk phase of molecules that have stronger intermolecular interactions. Figure D3.5.3 illustrates this concept of molecular interactions between molecules within bulk phases and at the interface. The illustration also offers an explanation as to why surface tension (gas-liquid) is usually higher than interfacial tension (liquid-liquid). At interfaces there is an additional (balancing) contribution from molecules in the second liquid phase. In case of a surface, the second phase consists of a gas that contains a much lower number of molecules within the same volume at the same pressure. The interaction between molecules of the two phases is fairly small because of the low concentration of gas molecules in the vicinity of the interface.

Thermodynamic Relations in Systems with Interfaces

As previously mentioned, interfacial tension is a key parameter primarily because it can be used to derive a large number of additional thermodynamic relationships that in turn can be used to completely describe the state of an interfacial system under various conditions. These relationships offer substantial insight into the nature of multiple-phase systems. A set of key equations are derived below. Assuming that the system is in thermodynamic equilibrium—that is, the pressure, temperature, and composition are constant within the system boundaries—the inner energy is given as:

$$U = TS - PV + \gamma A + \sum_i \mu_i n_i$$

Equation D3.5.10

where μ_i is the chemical potential of component i, n_i is the concentration of species i, and S is entropy. From this, the following standard thermodynamic quantities emerge:

$$H \equiv U + PV = TS + \gamma A + \sum_i \mu_i n_i$$

Equation D3.5.11

$$F \equiv U - TS = -PV + \gamma A + \sum_i \mu_i n_i$$

Equation D3.5.12

$$G \equiv U + PV - TS = \gamma A + \sum_i \mu_i n_i$$

Equation D3.5.13

where H is the enthalpy, F the Helmholtz free energy, and G the Gibbs free energy. These basic equations can be used to derive explicit expressions for these quantities as they apply

to interfaces. Based on the Gibbs definition of the interface, U^{σ} has in fact become a measurable quantity. It would therefore be of considerable advantage to express the other thermodynamic quantities (Equations D3.5.11 through D3.5.13) in terms of the inner energy. Equation D3.5.11 stated that the enthalpy can be expressed as a function of the inner energy. For the interfacial parameters it can therefore be stated that $H^{\sigma} = U^{\sigma} + PV^{\sigma}$. According to the Gibbs definition of the interface, however, $V^{\sigma} = 0$, which renders the above definition useless, unless one wants to set the inner energy and the enthalpy equal by definition (which is not a particularly useful concept). Instead, it is much more sensible to utilize the previous derivation of the interfacial work (Equation D3.5.9) and relate it to the enthalpy of the interface via:

$$H^{\sigma} \equiv U^{\sigma} - \gamma A$$

Equation D3.5.14

One can define the Helmholtz free energy F^{σ} and the Gibbs free energy G^{σ} of the interface in a similar fashion:

$$F^{\sigma} \equiv U^{\sigma} - TS^{\sigma}$$

Equation D3.5.15

$$G^{\sigma} \equiv U^{\sigma} - TS^{\sigma} - \gamma A$$

Equation D3.5.16

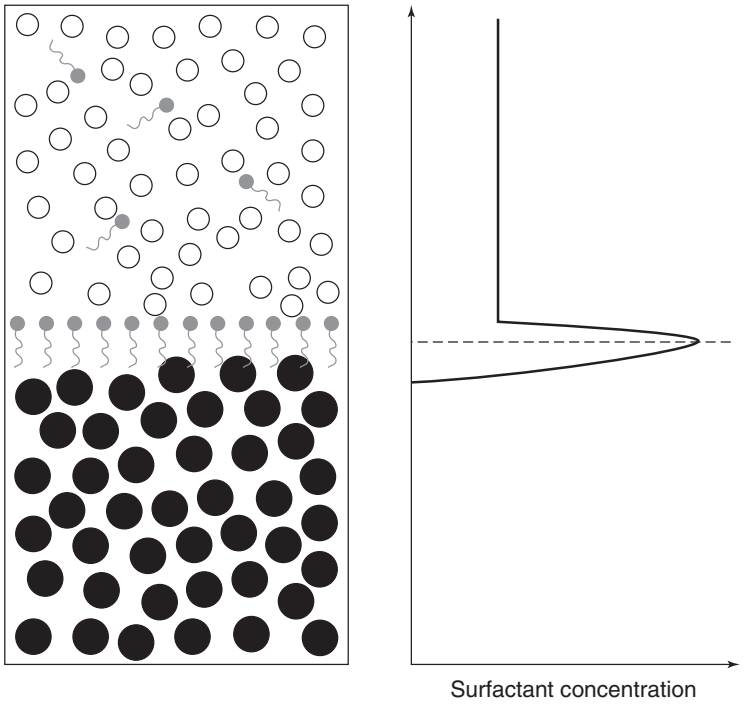

Figure D3.5.4 Interfacial excess concentration of a two-phase system containing surface-active material. The concentration of surfactant at the interface is larger than the concentration within either of the two bulk phases.

In a second step, the former Gibbs definition of the interface (Equation D3.5.1), which explicitly contains the inner energies of the bulk phases α and β, gives:

$$U^{\sigma} = TS - PV + \gamma A + \sum_i \mu_i n_i - TS^{\alpha} + PV^{\alpha} - \sum_i \mu_i n_i^{\alpha} - TS^{\beta} + PV^{\beta} - \sum_i \mu_i n_i^{\beta}$$

$$= T(S - S^{\alpha} - S^{\beta}) - P(V - V^{\alpha} - V^{\beta}) + \sum_i \mu_i (n_i - n_i^{\alpha} - n_i^{\beta}) + \gamma A$$

$$= TS^{\sigma} + \gamma A + \sum_i \mu_i n_i^{\sigma}$$

Equation D3.5.17

Using Equation D3.5.17 to substitute the expression for U^{σ} in Equations D3.5.14, D3.5.15, and D3.5.16, the final fundamental thermodynamic integral expressions for the state of the interface are obtained:

$$H^{\sigma} = TS^{\sigma} + \sum_i \mu_i n_i^{\sigma}$$

Equation D3.5.18

$$F^{\sigma} = \gamma A + \sum_i \mu_i n_i^{\sigma}$$

Equation D3.5.19

$$G^{\sigma} = \sum_i \mu_i n_i^{\sigma}$$

Equation D3.5.20

While the derivation of these quantities seems at first a bit of a mathematical card trick without a real application, interfacial scientists do utilize these equations on a day-to-day basis. The Gibbs adsorption equation that relates interfacial tension to the interfacial coverage is a perfect example of a thermodynamic relationship that can be obtained from

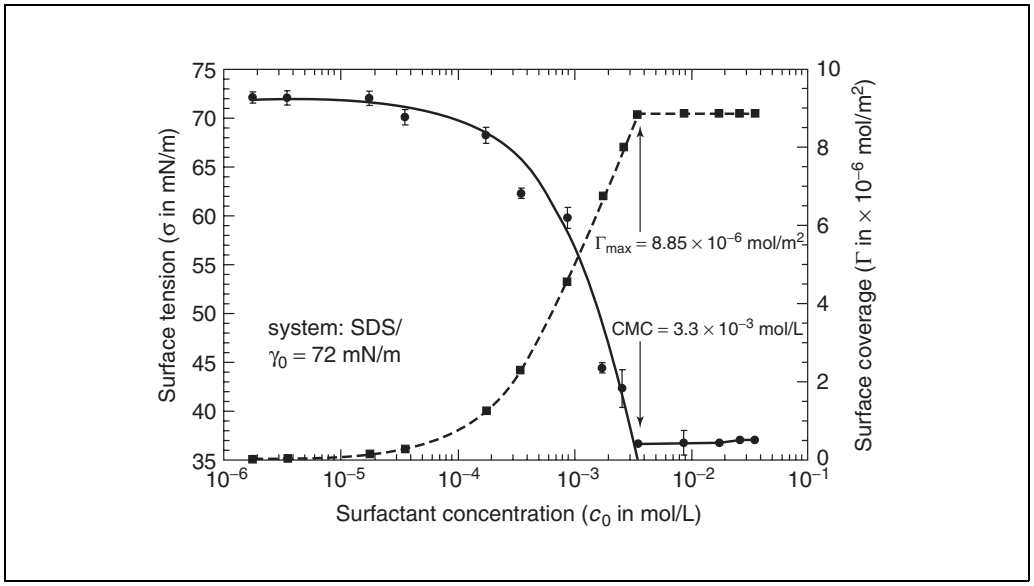

Figure D3.5.5 Equilibrium surface tension of sodium dodecyl sulfate (SDS) at the air-water interface as a function of surfactant concentration. The corresponding surface coverage was calculated using the Gibbs absorption equation (Eq. D3.5.26).

the above derived integral expressions. Another interesting insight that can be gained from this mathematical exercise is the fact that there is an entropic contribution to the interfacial energy:

$$\left(\frac{\partial \gamma}{\partial T}\right)_{A,n_i^\sigma=const} = -\left(\frac{\partial S^\sigma}{\partial A}\right)_{T,n_i^\sigma}$$

Equation D3.5.21

As can be seen, the magnitude of the entropic contribution can be directly evaluated by measuring the temperature dependence of the interfacial tension. A detailed discussion on the further use of these equations can be found elsewhere (Baszkin and Norde, 2000).

INTERFACIAL PROPERTIES

Interfacial Coverage, the Gibbs Equation, and Adsorption Isotherms

The direct contact between nonpolar and polar substances is energetically highly unfavorable and phases therefore have the tendency to minimize the contact area between the two molecular species. One would therefore assume that nature would obey the thermodynamic tendency to create as few interfaces as possible due to the high instability that is associated with the formation and maintenance of interfacial boundaries. On the other hand, many biological and chemical reactions simply could not proceed without the presence of an interface. Nature has therefore come up with an elegant solution to this problem. A variety of compounds exist, the so-called surfactants, that are able to interact simultaneously with both nonpolar and polar molecules. The name surfactant is an abbreviation that stands for surface-active substances. Surfactants are amphiphilic molecules—i.e., one part of the molecule has a hydrophilic character while a second part of the molecule possesses a hydrophobic character. The hydrophilic part is usually referred to as the headgroup of the surfactant, whereas the hydrophobic group is called the tail. The amphiphilic character of these compounds is a determinant in the rate at which surfactants adsorb to interfaces. A surfactant can be defined as "a chemical substance that adsorbs preferably at surfaces/interfaces if dissolved in a liquid thereby causing a change in the physicochemical or chemical properties of the system" (Schick, 1967). Many

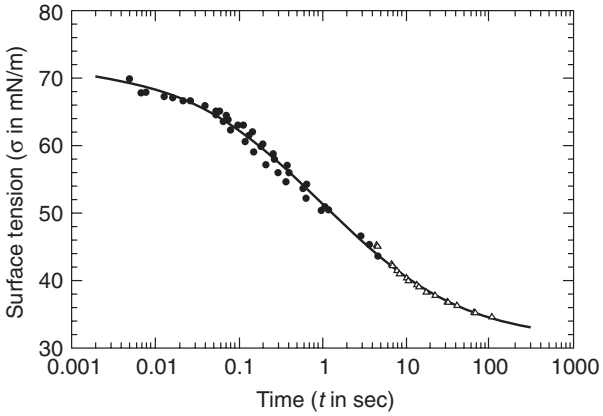

Figure D3.5.6 Adsorption kinetics of a small molecule surfactant. Surface tension of polyoxyethylene (10) lauryl ether (Brij) at the air-water interface decreases as time of adsorption increases. Brij concentration is 0.1 g/liter, as measured by the drop volume technique and the maximum bubble pressure method (*UNIT D3.6*).

surfactants reorient after adsorption from the bulk phase at the interface. Interfacial films are therefore highly ordered systems.

It is now time to reconsider the simple case of a two-phase system that contains two different types of molecules. If molecules of phase α are polar and molecules of phase β are nonpolar, the introduction of amphiphilic molecules that are capable of associating with either one of the two bulk phase molecules will result in an accumulation at the interface. Hence, these molecules will have a true excess concentration at the interface. Figure D3.5.4 illustrates that once surfactants adsorb at interfaces, the concentration within the interface may be larger than in any of the other phases. In order to predict the influence that these adsorbed surfactant molecules can have on the properties of the bulk system, interfacial chemists must be able to quantify the number of molecules that are adsorbed at the interface, that is, they must be able to measure the interfacial coverage. Unfortunately, it is extremely difficult, if not impossible, to directly measure the concentration of surface-active molecules adsorbed in a two-dimensional plane. This is where the thermodynamic concepts discussed earlier prove to be very useful, because a relationship between the interfacial coverage (G) and the interfacial tension (γ) can be derived.

Equation D3.5.13 illustrated that the free energy of an interfacial system can be expressed in terms of the interfacial tension and chemical potential of the overall system. A simple differentiation or alternatively the reutilization of the definition of the interfacial tension used in Equation D3.5.7 at constant pressure and temperature yields:

$$dG = \gamma dA + \sum_i \mu_i dn_i$$

Equation D3.5.22

Integrating and differentiating Equation D3.5.22 according to Eulers equation leads to:

$$dG = \gamma dA + A d\gamma + \sum_i \mu_i dn_i + \sum_i n_i d\mu_i$$

Equation D3.5.23

Combining Equation D3.5.22 with Equation D3.5.23 yields:

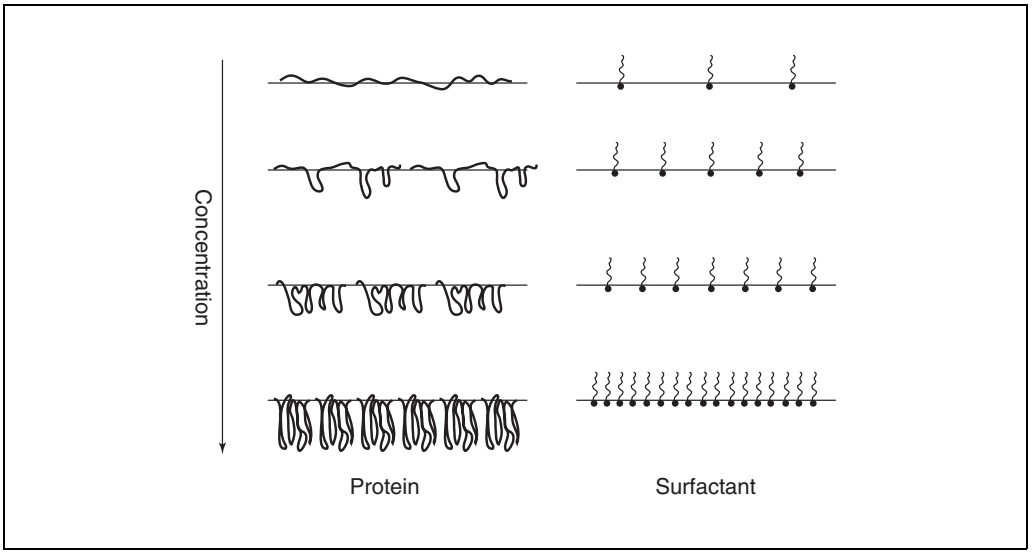

Figure D3.5.7 Molecular reorientation of surface-active molecules at the interfaces. (Left) Protein molecules unfold less extensively at interfaces as the protein concentration increases. (Right) Orientation of small-molecule surfactants is independent of surfactant concentration in the bulk phase.

$$Ad\gamma = -\sum_i n_i d\mu_i$$

Equation D3.5.24

The interfacial coverage Γ was originally introduced as a simple means to define the position of the Gibbs dividing plane:

$$\Gamma_i = \frac{n_i}{A}$$

Equation D3.5.25

where n_i is the excess number of moles of component *i* at the interface and *A* is the surface area. This definition can now be used to simplify Equation D3.5.24. After division through the interfacial area, the famous Gibbs adsorption equation emerges:

$$d\gamma = -\sum_i \Gamma_i d\mu_i$$

Equation D3.5.26

For a binary system that contains only one surfactant at dilute concentration, the chemical potential can be replaced with the logarithm of the concentration of surfactant (*c*), the temperature (*T*), and the universal gas constant (*R*) to obtain:

$$\Gamma = -\frac{1}{RT}\left(\frac{d\gamma}{d\ln c}\right)$$

Equation D3.5.27

From a practical point of view, this means that the concentration of molecules within the interface can be determined by simply measuring how the interfacial tension decreases as more and more surface-active material is added to the bulk phase. With Equation D3.5.25 comes a means with which to directly obtain information about the nature of the interface. Figure D3.5.5 shows the example of an application of the Gibbs adsorption equation. The surface tension of sodium dodecyl sulfate (SDS), a low-molecular-weight anionic (negatively charged) surfactant was measured as a function of the surfactant concentration. Equation D3.5.27 was then used to calculate the corresponding interfacial excess concentration. Figure D3.5.5 also illustrates another interesting phenomenon, that interfacial coverage eventually reaches a maximum. At this point the interface is fully covered with surfactant molecules. If more surfactant molecules were aded to the system, the molecules would no longer adsorb at interfaces and would instead form self-assembled structures such as micelles or vesicles. The specific concentration at which micelles are formed is referred to as the critical micellar concentration (CMC).

Beside the theoretically derived Gibbs adsorption isotherm, a large number of models have been developed that empirically describe a relationship between the interfacial coverage, the surface tension, and the surfactant concentration in the bulk phase. These adsorption isotherms are known under the names of the authors that first described them—i.e., the Langmuir, Frumkin, or Volmer isotherms. A complete mathematical description of these isotherms is beyond the scope of this unit and the reader is encouraged to consult the appropriate literature instead (e.g., Dukhin et al., 1995).

Adsorption Kinetics: Diffusion and Kinetic Controlled Models

It should be noted that food systems are not static systems that can necessarily be treated as thermodynamic systems in equilibrium. During food manufacturing processes, new interfaces are constantly created or destroyed, for example, in unit operations such as grinding, mixing, homogenization, or agglomeration. Food systems are highly dynamic. Environmental influences such as temperature, pressure, or humidity fluctuations can cause the structure of the food system to vary over time. From a surface science point of view, interfaces in food systems are therefore in constant flux as well. There is a continuous exchange of molecules between bulk and interface. This molecular exchange is associated with a specific rate, since adsorption (or desorption) of surface-active substances out of a bulk phase does not occur instantaneously. In many food application processes, new interfaces are created. These interfaces are initially not covered by surface-active material. The interfacial tension will thus be quite high. As time proceeds, surface-active material will adsorb at these interfaces and decrease the interfacial tension, thus stabilizing the system. A finite time is required for surfactant molecules to diffuse to the interface. The kinetics of this process will affect chemical and physical processes in the food system because the concentration and composition of surface-active substances will now vary with time. In the case of proteins, it can actually take several hours for a previously uncovered interface to be fully covered with protein molecules. Figure D3.5.6 shows an example of experimentally obtained dynamic surface tension data. The small molecule surfactant that was used in this study (Brij), adsorbs rapidly at the fresh interface and reaches equilibrium after 200 to 300 sec.

The objective in this section is to derive a mathematical model that can be used to extract the rate of adsorption from experimentally obtained dynamic surface tension data. Various investigators have speculated that the mechanism of surfactant adsorption involves two subsequent steps:

1. Transport of surfactant molecules from the bulk to a "subsurface" layer located in the direct vicinity of the interface.

2. Incorporation of surfactant molecules into the interface (most likely via a "flip-flop" mechanism).

Ward and Tordai (1946) proposed a nonlinear volterra integral function to establish a relationship between the dynamic surface coverage [$\Gamma(t)$] and the concentration of surface-active material in the subsurface [$c(0,t)$] for freshly formed surfaces:

$$\Gamma(t) = 2\sqrt{\frac{D}{\pi}}\left[c_0\sqrt{t} - \int_0^{\sqrt{t}} c\left(0, t-\tau\right) d\sqrt{\tau} \right]$$

Equation D3.5.28

where D is the diffusion coefficient and t is time. The Ward-Tordai equation cannot easily be solved, but one can calculate two asymptotical solutions for $t \to 0$ or $t \to \infty$. The asymptotical solution for $t \to \infty$ was published by Joos and Rillaerts (1981):

$$\Gamma(t) = 2\sqrt{\frac{2Dt}{7\pi}}\left[c_0 - c\left(0,t\right) \right]$$

Equation D3.5.29

Using Gibbs adsorption isotherm, an expression for the time-dependent decrease of the surface tension is obtained:

$$\gamma(t) = \gamma_\infty + \frac{RT\Gamma^2}{c_0}\sqrt{\frac{7\pi}{12Dt}}$$

Equation D3.5.30

The short-term solution was calculated by Bendure (1971):

$$\Gamma(t) = 2c_0\sqrt{\frac{Dt}{\pi}}$$

Equation D3.5.31

Inserting Gibbs equation (Eq. D3.5.27) yields the following expression:

$$\gamma(t) = \gamma_\infty - 2RTc_0\sqrt{\frac{Dt}{\pi}}$$

Equation D3.5.32

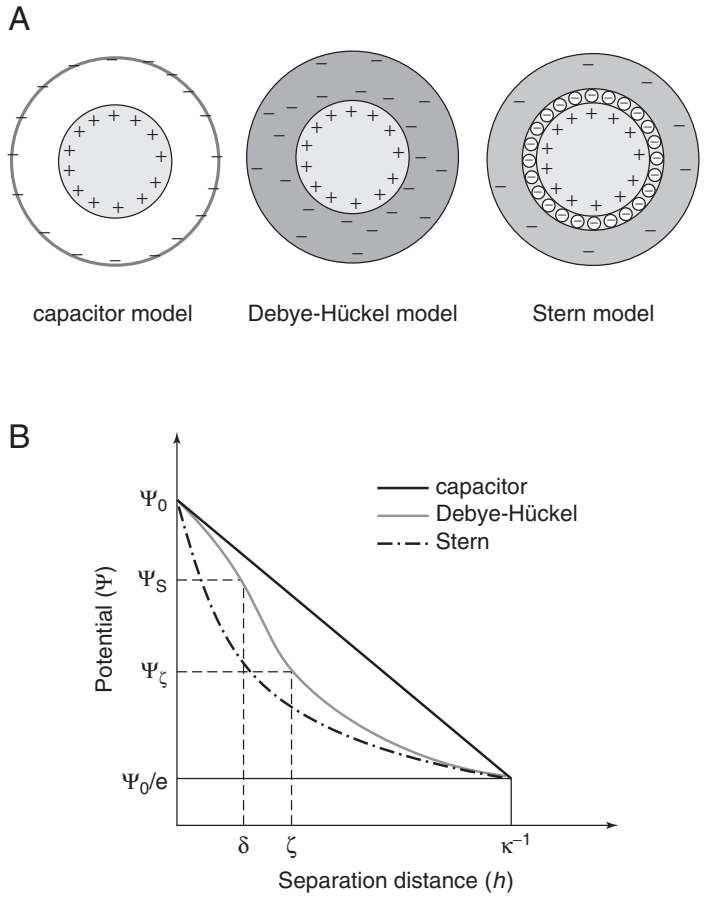

Figure D3.5.8 (**A**) Three possible models for systems (spherical particle) with an electrical double layer. (**B**) Corresponding electrical potential as a function of the separation distance.

Equations D3.5.30 and D3.5.32 are both very valuable. They state that the rate of adsorption can be obtained from plots of the interfacial tension versus either $t^{1/2}$ (for $t \rightarrow 0$) or $1/t^{1/2}$ (for the long-term solution $t \rightarrow \infty$). With these two equations the tool to extract the adsorption rate from experimentally obtained surface tension-time curves is at hand. It should be noted that instead of the Gibbs model, one could use one of the previously mentioned adsorption isotherms such as the Langmuir adsorption isotherm to convert interfacial tension to interfacial coverage data. The adsorption isotherms may be obtained by fitting equilibrium surface tension data versus surfactant concentration.

As a final remark, it should be mentioned that there is still ongoing discussion about the mechanisms and appropriate theoretical models of adsorption kinetics. Recent publications suggest that under special circumstances a third step, the possible reorientation of molecules at the interface, may determine the overall rate. Reorientation will play a less dominant role in the adsorption of small-molecule surfactants, but it can significantly affect the rate of adsorption of large macromolecules such as proteins. Figure D3.5.7 illustrates that in the case of protein adsorption an additional concentration-dependent unfolding term should indeed be introduced into the Ward-Tordai model. At small concentrations, the adsorbed proteins will begin to unfold, thereby assuming an energetically lower state, reducing the interfacial tension further. At higher concentrations, the interface will be rapidly saturated with tightly packed proteins. These proteins will not be able to unfold. Thus, the rate of adsorption appears to be faster.

The Electrical Double Layer, Interfacial Charge Density, and Zeta Potential

Interfaces can carry an electrical charge due to the adsorption of molecules that either are ionic or are capable of being ionized (e.g., proteins, certain polysaccharides, or ionic surfactants). In interfacial chemistry, the presence of an interfacial layer that carries a charge is of utmost importance since many reactive species or compounds that are capable of catalyzing a reaction can carry a charge as well. The interaction of reactant and catalyst will thus influence the overall reaction. If, for example, the catalyzing agent and interfacial layer carry a similar charge, then the repulsive electrostatic interaction will prevent the catalyst from coming into close contact with the substrate, and the kinetics of the chemical reaction will be greatly reduced. On the other hand, if the catalyst and the interfacial layer carry an opposite charge, then the catalyst can actually be physically bound to the layer and the concentration within the vicinity of the interface is therefore much greater than one would ordinarily expect. As a result, the rate of the reaction will be increased. The goal of this section is to introduce some fundamental electrochemical properties of interfaces and highlight their importance in food chemistry.

As previously mentioned, molecules that are present within the interface may be able to bind or release electrons from the outer electron hull that surrounds the positively charged proton-neutron core—i.e., they can be ionized. In systems with interfacial boundaries containing ions that carry a charge, a spatial distribution of counter ions surrounding the interface will develop. The number of counter ions will decrease as the distance from the interface increases. The counter ion atmosphere is also referred to as the ion cloud. The

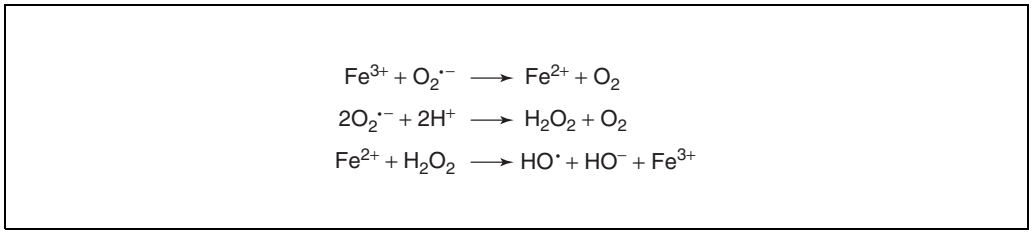

Figure D3.5.9 Initiation of lipid oxidation through formation of active oxygen species via metal-catalyzed reactions.

two regions, charged interface and surrounding ion cloud, are known as the electrical double layer. Since the overall charge of the system has to be zero, the charge at the interface itself must balance the combined charge of the ion atmosphere. The area-specific excess charge at the interface is called the interfacial charge density σ*:

$$\sigma^* = \frac{q}{A}$$

Equation D3.5.33

where A is the interfacial area and q the total charge at the interface. The concept of an excess charge has a lot of similarities with the previously discussed concept of an excess concentration, only that in this particular case it refers to the concentration of electrons instead of the concentration of surface-active molecules that are present within the interfacial boundary. The next logical question is how is the interfacial charge density determined? Here the researcher is faced with the same problem as when trying to determine the interfacial coverage—i.e., the parameter of interest can not be directly measured. Instead, a model is needed that establishes a relationship between the interfacial charge density and a parameter that is directly measurable.

Based on the previous description of the double layer, it is logical to assume that a direct relationship between the absolute charge at the interface and the concentration of ions in the vicinity of the interface exists. Indeed, several models have been developed in the past that describe the ion concentration as a function of the actual surface charge at a specific distance x from the interface. Furthermore, the famous Nernst equation, which is the basis for understanding many electrochemical reactions, proves to be helpful as it relates the ion concentration to a quantity called the electrical potential (ψ). The electrical potential is the work (W) required to move a unit charge (q) through the electrical field:

$$\psi = \frac{W}{q}$$

Equation D3.5.34

The electrical potential is a function of distance x from the interface and can be measured at a specific distance z from the interface. The electrical potential as measured by means of electrophoretic mobility is called the zeta potential (ψ_Z). With the zeta potential, it is possible to calculate the actual interfacial charge. However, the use of the appropriate model is essential to obtaining the correct interfacial charge and can be evaluated using

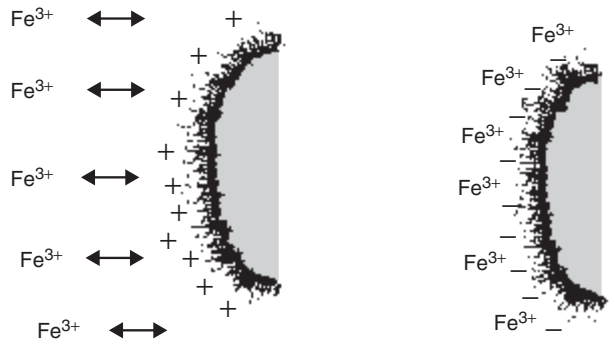

Figure D3.5.10 Effect of charge of interfacial membrane on location and distribution of metal catalyst.

three important equations described below. A more extensive derivation and discussion of this topic can also be found in the literature (Hiemenz and Rajagopalan, 1997)

The capacitor model

The earliest model that describes the electrochemical potential as a function of the distance from the interface is the capacitor model. This model is used to derive the fundamental equations required to perform the necessary calculations to obtain the surface charge. As other models follow a similar scheme, only the electrochemical potential functions are introduced here.

The capacitor model simply assumes that *all* ions in the surrounding cloud are located in a thin plane or shell located at a specific distance δ from the interface. The combination of charged interface and charged plane or shell resembles the basic design of a conductor with a difference in the electrical potential of $\Delta\psi$ between the two plates. Coulomb's law, which was first mentioned in 1785, quantifies the force (F) that acts between two charges (q_1 and q_2) separated by a distance (x):

$$F_{Coulomb} = \frac{1}{4\pi\varepsilon_0} \cdot \frac{q_1 q_2}{\varepsilon_r x^2}$$

Equation D3.5.35

Each of these charges creates an electrical field (E) with a magnitude that is given by the force per unit charge:

$$E = \frac{F}{q}$$

Equation D3.5.36

Based on Coulombs law, the field strength that develops as two identical charges q are brought in close contact (i.e., at a specific separation distance x) becomes:

$$E = \frac{1}{4\pi\varepsilon_0} \cdot \frac{q}{\varepsilon_r x^2}$$

Equation D3.5.37

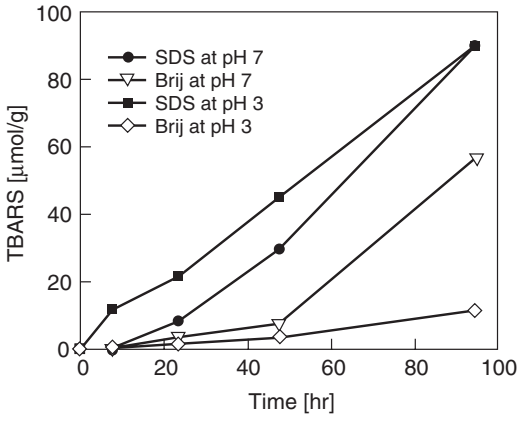

Figure D3.5.11 Time-dependent TBARS development for a 2% (w/v) fish oil emulsion stabilized by either SDS or Brij at pH 3 and 7. Adapted from Mei et al. (1999) with permission from the American Chemical Society.

Now the interfacial charge density is defined as the overall interfacial charge per unit area:

$$\sigma^* = \frac{q}{A}$$

Equation D3.5.38

The strength gradually decreases as the distance to the center of the charge increases. A particle that is in the vicinity of such an electrical field will experience a force that is simply the negative gradient of the electrical potential, which provides an elegant explanation as to why work is needed to move a charge through an electrical field. Thus, a second derivation of the electrical field strength emerges:

$$E = \frac{d\psi}{dx}$$

Equation D3.5.39

This an important step in determining the charge at the interface. If Equations D3.5.37 and D3.5.39 are combined, an expression that directly relates the interfacial charge to the electrical potential is obtained. In the simplified capacitor model, a linear increase in the potential between interface and ion shell was assumed. Hence, the $d\psi/dx$ differential in Equation D3.5.39 can be replaced with the absolute difference $\Delta\psi/\delta$, where δ is the distance between interface and ion shell. Using the total differential, the desired relationship is obtained.

The Debye-Hückel model

The Debye-Hückel theory was developed to extend the capacitor model and is based on a simplified solution of the Poisson equation. It assumes that the double layer is really a diffuse cloud in which the potential is not a discontinuous function. Again, the interest is in deriving an expression for the electrical potential function. This model states that there is an exponential relationship between the charge and the potential. The distribution of the potential is:

$$\psi = \psi_0 \exp(-\kappa x)$$

Equation D3.5.40

where κ is the so-called Debye length:

$$\kappa = \sqrt{\frac{e^2 \sum_{n=1}^{i} z_i^2 n_{i,0}}{\varepsilon k T}}$$

Equation D3.5.41

where $n_{i,0}$ is the number of ions of type i per unit volume far away from the surface, and z_i is the valency of the ion species. One of the restrictions of the Debye-Hückel approximation is that it is only valid for low potentials. κ is a very useful parameter indicating how quickly the potential decreases with increasing distance to the surface. κ^{-1} is often referred to as the thickness of the double layer. As can be seen from Equation D3.5.41, κ depends on the electrolyte concentration and valency, which explains why electrostatic repulsion between two particles (e.g., emulsion droplets) decreases upon the addition of

ions to the aqueous phase of the emulsion. A better model was developed by Guoy and Chapman based on the complete solution of the Poisson equation. The Guoy and Chapman model is also valid for higher potentials, which is a limitation of the Debye-Hückel formula. Nevertheless, the above equation is usually sufficient to describe the situation of an electrical double layer in interfacial systems.

The Stern model

One major drawback of both the Debye-Hückel and the Guoy and Chapman models is that they do not take into account that real ions have a finite radius—i.e., they are not infinitesimally small. From a practical point of view, this means that only a finite number of ions can be located in the immediate vicinity of the interface. Stern argued that there must be a layer of ions that are directly attached to the interface. In this Stern layer, the potential decreases linearly in accordance with the capacitor model. Outside the Stern layer it decreases exponentially as described by the Debye-Hückel model. This also accounts for the case of a complete charge reversal (e.g., the adsorption of multivalent ions to the interface).

As indicated earlier, the quantity that is actually measured is the zeta potential, which can be obtained from the measurement of the velocity of a charged particle or molecule in an electric field. Velocity per unit field is defined as mobility of the ion and is directly related to the zeta potential, which is the potential at the plane of shear. In the case of a spherical particle or molecule that carries a surface charge, the zeta potential is given as:

$$\zeta = \frac{q}{4\pi\varepsilon_0\varepsilon_r R} \cdot \frac{\kappa^{-1}}{R + \kappa^{-1}}$$

Equation D3.5.42

In conclusion, it can be said that the procedure to determine the interfacial charge includes first the measurement of the zeta potential, after which the correct model for the electrical potential is applied. From the model one can easily calculate the interfacial charge or the interfacial charge density. Figure D3.5.8A illustrates the differences between the three different models using the example of a spherical particle that carries a negative charge. The surface potential for these three different models is plotted as a function of the separation distance from the interface (Figure D3.5.8B). This illustrates how important it is to remember that a model is used to obtain the actual surface charge density. If the wrong model is used, the calculated surface charge will be dramatically different. For example, assume that multivalent ions are present in the bulk phase. In this case, a charge reversal may occur in the bound ion layer. It would therefore be theoretically possible to measure a positive zeta potential even though the interface itself may be negatively charged. Thus, the composition of the system in question (and particularly the types of ions that are present) influences the decision about which model should be used, a fact that needs to be considered prior to zeta potential measurements.

CASE STUDY: LIPID OXIDATION OF EMULSIONS

The case of lipid oxidation in an emulsified system is a perfect example to illustrate the importance of interfacial properties in food chemistry. The goal of this case study is not to completely describe the very complex mechanisms of lipid oxidation in emulsions. Indeed, many investigators over the past years have focused on this research area. Instead, the key interfacial parameters that influence lipid oxidation in emulsions are emphasized.

Autoxidation and Activation of Oxygen

Systems that contain lipids can undergo oxidative deterioration by reacting with molecular oxygen. The reaction of lipids with previously activated molecular oxygen through a self-catalyzing mechanism is known as autoxidation. The classical free radical autoxidation mechanism can in general be divided into four major steps: initiation, propagation, branching, and termination. Step one, initiation (i.e., the formation of free radicals), has been suggested to be the step that controls the kinetics of the reaction. Many studies have therefore focused on the mechanisms of oxygen activation. Extensive reviews are available on the role of reactive oxygen intermediates in biochemistry (e.g., Kanner, 1994). Two electron reactions of ground state dioxygen are thermodynamically unfavorable due to spin restriction of the two unpaired electrons. Stepwise activation through donation and acceptance of a single electron is energetically more favorable. The reaction yields several reactive oxygen species: superoxide anion radical ($O_2^{\cdot-}$), perhydroxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). The course of this reaction is influenced by a number of parameters such as temperature, pH, and particularly the presence of metal catalysts or salts. In a first step, the superoxide anion can be formed through ionizing radiation such as UV or chemical redox reactions with biological compounds. More importantly, the presence of transition metals such as iron or iron complexes can stimulate an entire series of reactions. Figure D3.5.9 presents an overview of three reactions that can produce free radicals. The first reaction shows the reduction of iron by the superoxide anion. Other reducing compounds such as ascorbic acid and thiols can also participate in this reaction. The second reaction shows the dismutation of the superoxide anion into hydrogen peroxide. In the third reaction, known as the Fenton reaction, reduced iron decomposes hydrogen peroxide into the extremely reactive hydroxyl radical (Decker and Hultin, 1992). Lipid oxidation can also be stimulated by a similar reaction with naturally preformed lipid hydroperoxides and ferrous iron to produce alkoxyl radicals.

The Role of the Interface in Lipid Oxidation

In many respects, the oxidation of lipids in emulsions follows the same mechanisms as in bulk oils. As for bulk oils, lipid oxidation is influenced by partial oxygen pressure and oxygen solubility, or the presence of antioxidants, salts, and other ingredients in the system. Contrary to bulk oils, however, the presence of an interfacial membrane or, more generally, a phase boundary will have a significant influence on the kinetics of the oxidative degradation of lipids (McClements and Decker, 2000). Not only does the phase boundary alter concentration of solutes such as antioxidants, prooxidants, and oxygen across the membrane due to different solubilities in the two phases (Huang et al., 1997), it also affects the kinetics of the mass transport of these species across the membrane. Hence the thickness and density of the interface in question will be of considerable importance. Model calculations of water-in-oil emulsions have shown that the ability of the membrane to retard these transport processes is often largely underestimated (Chaiyasit et al., 2000).

Probably far more important than the partitioning behavior is the fact that lipid oxidation in emulsions is *site specific*—i.e., most of the radicals are actually formed at or near the membrane. A close proximity to the interface is required to catalyze the reaction (Asua et al., 1989). Studies have shown that it is primarily the interfacial/subsurface concentration of transition metals that controls the rate of the reaction, while the concentration of transition metals in the bulk phase is secondary. This insight also provides a means to reduce the rate of the reaction. Experiments using a number of different antioxidants have in fact proven that lipid oxidation is most effectively inhibited if surface-active antioxidants are added. Antioxidants that are able to accumulate at the interface are closer to the site of lipid oxidation initiation and are therefore more efficient (Frankel et al., 1996).

Due to the site-specific mechanism, the charge of the membrane will play an important role as well. Positively charged transition metal ions are attracted to the negatively charged interfaces, thereby increasing the concentration of catalysts in the vicinity of the interface. The opposite is true for positively charged membranes. In fact, it has been shown that lipid oxidation in micelles stabilized by positively charged tetradecyltrimethylammonium bromide (TTAB) surfactant molecules proceeds at a much slower rate than lipid oxidation in negatively charged sodium dodecyl sulfate (SDS) micelles (Fujii et al., 1991). This concept is illustrated in Figure D3.5.10. It should also be noted that the addition of substances to the aqueous phase of emulsions that reduce the electrostatic repulsion will also have a significant influence on the stability against lipid oxidation. This may in fact contribute to the pH dependence of many lipid oxidation processes. Figure D3.5.11 shows results of lipid oxidation studies obtained by Mei et al. (1999) for the oxidative degradation of 2% (w/v) salmon oil emulsions stabilized by either SDS or polyoxyethylene-10-lauryl ether (Brij) at pH 7 and 3. Compared to Brij-stabilized emulsions, the SDS-stabilized emulsion had TBARS concentration that were five to six times greater at pH 3 and 7. Furthermore, these experiments also confirmed that environmental parameters (e.g., pH) that can affect the interfacial charge can indeed have a slight influence on the rate of the reaction (Mei et al., 1998a,b).

A special case exists if the surfactants used to stabilize the emulsion are proteins. Some proteins have antioxidative properties in solution due mainly to the presence of free sulfhydryl groups (Yamamoto and Omori, 1994). These groups can act as free radical scavengers and thereby inactivate some of the radical species that cause lipid oxidation. On the other hand, proteins are often capable of binding metal ions that are present in the aqueous phase (Baron et al., 1997), thereby increasing the metal concentration near the oil phase. The binding of transition metals can be important in polar lipid membranes (Fukuzawa and Fujii, 1992). The two effects of promoting and preventing oxidation may occur at the same time. The same can be true for metal chelators. Metal chelators with a high molecular weight and slow diffusion coefficients can inhibit lipid oxidation, as has been shown for xanthan (Shimada et al., 1992). However, EDTA can have the opposite effect on lipid oxidation due to the reduction of the electrostatic repulsion for positively charged membranes (Takenaka et al., 1991).

SUMMARY

In this unit, the basic interfacial properties that can affect chemical reactions in food systems were introduced. The quantities that were derived through straightforward application of thermodynamic concepts provide the theoretical framework for the development of the required experimental methods that can be found in *UNITS D3.6 & D3.7*. The case study of lipid oxidation in emulsions briefly highlights how dramatically the chemical and physical properties of food systems can be influenced by the presence of phase boundaries. It should be mentioned that in addition to the introduced parameters, there are a variety of other interfacial properties that may be of importance to food scientists, such as the rheological properties of interfacial membranes (Section H), which may directly influence the stability and formation of foams. For the interested reader, see Key References for literature that provides a wealth of additional information about interfacial phenomena. The field of interfacial chemistry is still under active investigation, and new insights emerge almost daily that advance the understanding of the role of interfaces in food chemistry.

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Dukhin et al., 1995. See above.

Excellent reference to gain an enhanced understanding of the fundamentals of the dynamics of adsorption phenomena at liquid interfaces. An in-depth theoretical description of methods to determine dynamic interfacial tensions is included in the book.

Hiemenz, P.C. and Rajagopalan, R. 1997. See above.

The basic reference book for beginner and advanced colloidal chemists. Includes a detailed discussion of intermolecular interactions such as Van der Waals interactions, steric interactions, and electrostatic interactions.

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Static and Dynamic Interfacial Tension Analysis

In this unit, the most commonly used protocols to determine static and dynamic interfacial tension will be discussed. As shown in *UNIT D3.5*, many chemical and enzymatic reactions that occur in heterogeneous food systems depend on the properties of the interface between the various homogeneous phases. Lipid oxidation in disperse systems, such as emulsions, can, for example, be accelerated or decelerated by adding emulsifiers that will modify the chemical and physical properties of the interface and affect mass transport processes (*UNIT D3.5*). Emulsifier molecules are surface active due to their amphiphilic nature. They adsorb readily at freshly formed interfaces until an equilibrium concentration is reached between emulsifier molecules at the interface and emulsifier molecules in the bulk phase. The concentration at the interface is referred to as the interfacial coverage. The rate at which emulsifiers adsorb at interfaces is known as the adsorption kinetics. Both these parameters are of considerable importance in food chemistry. Unfortunately, neither the interfacial coverage nor the adsorption kinetics of surface-active substances (surfactants) can be measured directly. Instead, the static (time-independent) or dynamic (time-dependent) interfacial tension is measured. The obtained data can then be used to derive the rate of adsorption and interfacial coverage through the application of thermodynamic relationships (*UNIT D3.5*). As a convention, the term "surface" will be used exclusively in this unit to denote the special case of an interface in which one of the phases consists of a gas.

This unit will introduce two fundamental protocols—the Wilhelmy plate method (see Basic Protocol 1 and Alternate Protocol 1) and the du Noüy ring method (see Alternate Protocol 2)—that can be used to determine static interfacial tension (Dukhin et al., 1995). Since the two methods use the same experimental setup, they will be discussed together. Two advanced protocols that have the capability to determine dynamic interfacial tension—the drop volume technique (see Basic Protocol 2) and the drop shape method (see Alternate Protocol 3)—will also be presented. The basic principles of each of these techniques will be briefly outlined in the Background Information. Critical Parameters as well as Time Considerations for the different tests will be discussed. References and Internet Resources are listed to provide a more in-depth understanding of each of these techniques and allow the reader to contact commercial vendors to obtain information about costs and availability of surface science instrumentation.

STRATEGIC PLANNING

The choice between the static methods (Wilhelmy plate method and the du Noüy ring method) should primarily be based on the properties of the system being studied, in particular, the surfactant. As mentioned in *UNIT D3.5*, the transport of surfactant molecules from the bulk to the surface requires a finite amount of time. Since static interfacial tension measurements do not yield information about the true age of the interface, it is conceivable that the measured interfacial tension values may not correspond to equilibrium interfacial tension values (i.e., the exchange of molecules between the bulk and the interface has not yet reached full equilibrium and the interfacial tension values are therefore not *static*). If the surfactant used in the experiment adsorbs within a few seconds, which is the case for small-molecule surfactants, then both the Wilhelmy plate method and the du Noüy ring method are adequate. If the adsorption of a surfactant requires more time to reach full equilibrium, then the measurement should not be conducted until the interfacial tension values have stabilized. Since interfacial tension values are continuously displayed with

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Table D3.6.1 Suitability of Different Techniques for Determining Static and Dynamic Interfacial Tension

Method	Liquid/liquid	Liquid/gas	Time range
Plate tensiometry	Good	Good	10 sec-24 hr
Ring tensiometry	Difficult	Good	30 sec-24 hr
Pendant drop	Good	Good	10 sec-24 hr
Drop volume	Good	Good	1 sec-20 min
Maximum bubble pressure[a]	Possible	Good	1 msec-100 sec
Inclined plane[a]	Bad	Good	50 msec-1 sec
Oscillating jet[a]	Bad	Good	1-10 msec

[a]Dukhin et al. (1995)

the Wilhelmy plate method, the operator can easily identify when the interfacial tension becomes static. If a du Noüy ring is used, measurements will have to be repeated at increasing time intervals, which can substantially increase the time required to conduct the experiments.

The Wilhelmy plate and du Noüy ring methods can both be used to obtain liquid/liquid and liquid/gas interfacial tension measurements (see Table D3.6.1). For liquid/liquid interfaces, setup is more difficult than for the dynamic methods (drop volume and drop shape) because the second, lighter fluid must be carefully layered on top of the first fluid without disrupting the meniscus formed between the plate and primary fluid. At the same time, the position of the plate/ring relative to the interface must be adjusted to account for the increasing buoyancy. In the author's experience, this is particularly difficult with the du Noüy ring, but can be done more easily and routinely with the Wilhelmy plate. Many laboratories, however, are more likely to have access to a du Noüy ring instrument, as it was the first research-grade instrument that was widely available for measuring interfacial tension.

Both dynamic methods (drop volume and drop shape techniques) enable the user to determine adsorption kinetics over a substantial period of time (up to several hours) and both instruments are available at comparable prices. In making a choice between the two instruments, the user should consider that the drop volume technique offers slight advantages in determining the adsorption kinetics of surfactants in the initial phase of the adsorption process (e.g., the first few seconds), but requires substantially more time to determine the dynamic interfacial tension at long adsorption times. Finally, the measurement frequency (i.e., the number of measurements per time interval) can be substantially higher using the drop shape method due to the fact that no drop detachment is necessary and the interfacial tension is instead calculated from the recorded shape of a drop (for more information, see Background Information).

The main advantage of the static methods is cost. The equipment needed to conduct the dynamic measurements is approximately five times as expensive as the equipment required for static measurements (~$25,000 for a drop shape and drop volume analyzer versus ~$5,000 for du Noüy and Wilhelmy instruments). This is due to the additional capability of the former instruments to determine not only interfacial tension values but also the corresponding age of the interface. For more information on equipment, costs, and suppliers, see Internet Resources.

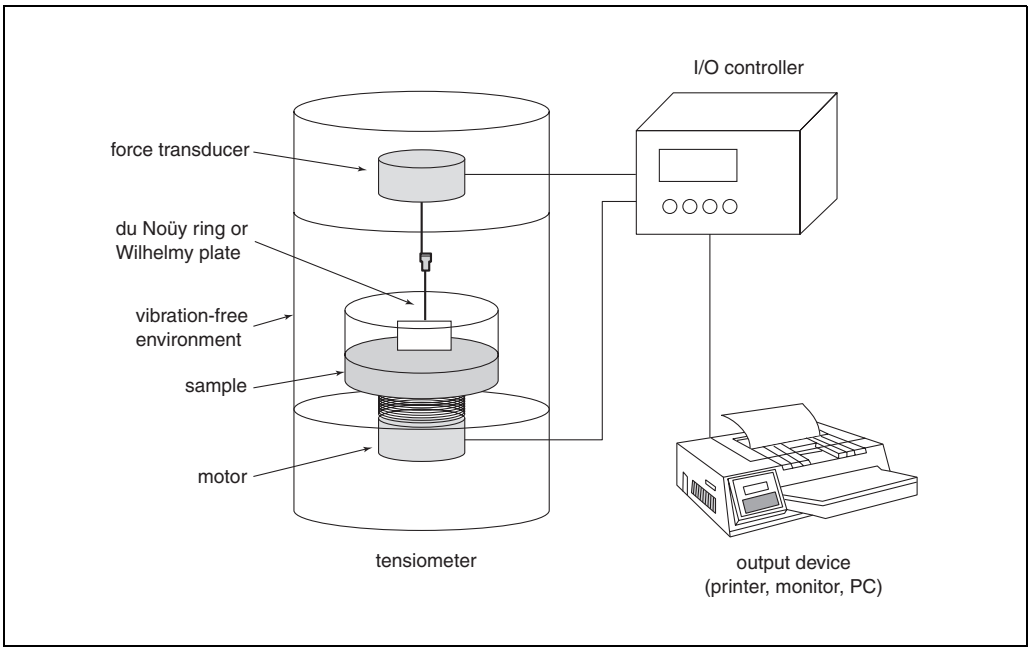

Figure D3.6.1 General principle of direct force-transducer-based tensiometers (Wilhelmy plate and/or du Noüy ring method). I/O is input/output.

STATIC INTERFACIAL TENSION DETERMINATION AT A LIQUID/GAS INTERFACE BY THE WILHEMY PLATE METHOD

BASIC PROTOCOL 1

The basic setup to determine static interfacial tension based on either the Wilhelmy plate method or the du Noüy ring method (see Alternate Protocol 2) is shown in Figure D3.6.1. It consists of a force (or pressure) transducer mounted in the top of the tensiometer. A small platinum (Wilhelmy) plate or (du Noüy) ring can be hooked into the force transducer. The sample container, which in most cases is a simple glass beaker, is located on a pedestal beneath the plate/ring setup. The height of the pedestal can be manually or automatically increased or decreased so that the location of the interface of the fluid sample relative to the ring or plate can be adjusted. The tensiometer should preferably rest on vibration dampers so that external vibrations do not affect the sensitive force transducer. The force transducer and motor are connected to an input/output control box that can be used to transmit the recorded interfacial tension data to an external input device such as a monitor, printer, or computer. The steps outlined below describe measurement at a liquid/gas interface. For a liquid/liquid interface, see the modifications outlined in Alternate Protocol 1. Other variations of the standard Wilhelmy plate method exist (e.g., the inclined plate method), which can also be used to determine static interfacial tension values (see Table D3.6.1).

Materials

~1 mM chromic acid, optional
Ultrapure water (for calibration)
Sample solution (surface-active liquid)

Sample container (250- or 500-ml glass beaker)
Bunsen burner
Forceps
Tensiometer with Wilhelmy plate (Figure D3.6.1; available from Lauda, Kruss, or Rheometric Scientific)

1. Clean sample container (250- or 500-ml beaker) using, e.g., highly diluted (~1 mM) chromic acid and rinse extensively with ultrapure water to completely remove any remaining acid.

   A 250- or 500-ml beaker may be used here. It is important that the surface area of the beaker is large enough so that once the Wilhelmy plate is introduced, space remains between the plate and the side walls of the beaker to prevent additional capillary forces from the walls acting on the plate. A beaker diameter of 7 to 8 cm and a height of ~5 to 6 cm are recommended.

   Cleaning with chromic acid may not always be necessary and depends largely on the previous contamination of the sample container. However, it is advisable to use chromic acid in the case of protein tensiometry, since proteins have a tendency to quickly adsorb and slowly desorb from glass surfaces.

2. Ignite a Bunsen burner and, using forceps to hold the rod to which the Wilhelmy plate is attached, briefly insert the Wilhelmy plate into the flame (2 to 3 sec) to remove any contamination from the plate.

   IMPORTANT NOTE: *Do NOT overheat the plate to the point that it glows white. Extensive heating can cause the plate to deform, which will reduce the accuracy of the measurement. Overheating can cause the plate holder to melt, requiring the platinum plate to be replaced, which is often associated with considerable costs.*

3. Lower the pedestal and place the glass beaker containing sample on the test stand.

4. Using forceps, attach the clean platinum Wilhelmy plate to the hook that is connected to the force transducer or pressure sensor mounted in the top of the tensiometer. Do not touch the plate, and make sure that the plate does not yet touch the surface of the sample.

   Fats, oils, and other substances that are embedded in human skin will contaminate the plate and invalidate the measurements.

5. Raise the pedestal with the sample solution until the plate touches the interface and a meniscus between the plate and the liquid interface is formed.

6. Slowly lower the pedestal until the bottom edge of the plate is at or above the level of the interface but is still attached to the meniscus.

   If the meniscus detaches, raise the pedestal again until the plate touches the interface. Then wait ~1 min to allow excess liquid to drain and repeat the procedure.

7. Lower the pedestal until the plate just detaches from the interface and wait until all plate movement has stopped. Zero (tare) the force transducer or pressure sensor.

8. Raise pedestal slowly. Stop as soon as the plate and the liquid interface touch and the meniscus between plate and interface are again formed.

9. Wait for the displayed interfacial tension value to stabilize and record the value.

ALTERNATE PROTOCOL 1

STATIC INTERFACIAL TENSION DETERMINATION AT A LIQUID/LIQUID INTERAFACE BY THE WILHEMY PLATE METHOD

The Wilhelmy plate method can also be used to determine interfacial tension at a liquid/liquid interface by layering a second, less dense fluid on top of the primary fluid after the proper meniscus between the plate and the primary interface has been formed. In this case, it is crucial that the second fluid be applied carefully to avoid disrupting the primary interface. In addition, a small correction is necessary to account for the buoyancy that the plate experiences due to the presence of the second fluid.

Additional Materials *(also see Basic Protocol 1)*

Primary and secondary sample fluids

1. Set up the instrument and the primary fluid to form a meniscus as described (see Basic Protocol 1, steps 1 to 5).
2. Using a pipet, slowly add enough secondary (less dense) sample liquid to the beaker to cover the previously formed interface and particularly the meniscus between plate and primary liquid interface.

   In order to not disturb the previously formed interface, it is recommended that the secondary fluid is added by positioning the tip of the pipet against the side of the beaker and letting the secondary liquid run down. This yields a gentle fluid flow that slowly covers the primary interface.

3. Wait for the displayed interfacial tension value to stabilize and record the value. Correct the interfacial tension by using the correctional term in Equation D3.6.2 (see Background Information).

   To calculate the correctional term, the depth to which the plate is submerged in the second liquid, the geometry of the plate, and the density of the second liquid are required. It should be noted that the error introduced is in most cases <0.5 mN/m.

STATIC INTERFACIAL TENSION DETERMINATION AT A LIQUID/GAS INTERFACE BY THE DU NOÜY RING METHOD

ALTERNATE PROTOCOL 2

As in Basic Protocol 1, the steps below describe determination of interfacial tension for a liguid/gas interface (i.e., a sample consisting of a single surface-active solution). Although it is very difficult, the ring method can also be used to determine the interfacial tension between two liquid phases (for modifications see Alternate Protocol 1).

Additional Materials *(also see Basic Protocol 1)*

Tensiometer with du Noüy ring (Figure D3.6.1)

1. Set up the sample and instrument as described (see Basic Protocol 1, step 1 to 3) using the du Noüy ring in place of the Wilhelmy plate (step 2).

   As for the Wilhelmy plate, the du Noüy ring must not be overheated.

2. Using forceps, carefully attach the platinum ring to the hook that is connected to the force transducer mounted in the top of the tensiometer. Do not touch the ring with your fingers, and make sure that the ring does not yet touch the surface of the sample.

   Fats, oils, and other substances that are embedded in human skin will contaminate the ring and invalidate the measurements.

3. Wait until the interfacial tension shown on the electronic display no longer varies due to the movement of the ring, and zero (tare) the force transducer.
4. Raise the pedestal with the glass beaker containing the sample until the surface of the sample touches and wets the ring.
5. Keep raising the pedestal until the ring is completely submerged in the sample liquid.

   The electronic display will show a small positive force that remains because of the wetting of the vertical support of the ring.

6. Slowly begin to pull the ring out of the liquid by gradually lowering the sample beaker.

   When the upper surface of the ring touches the liquid, the surface tension shown on the display will begin to increase.

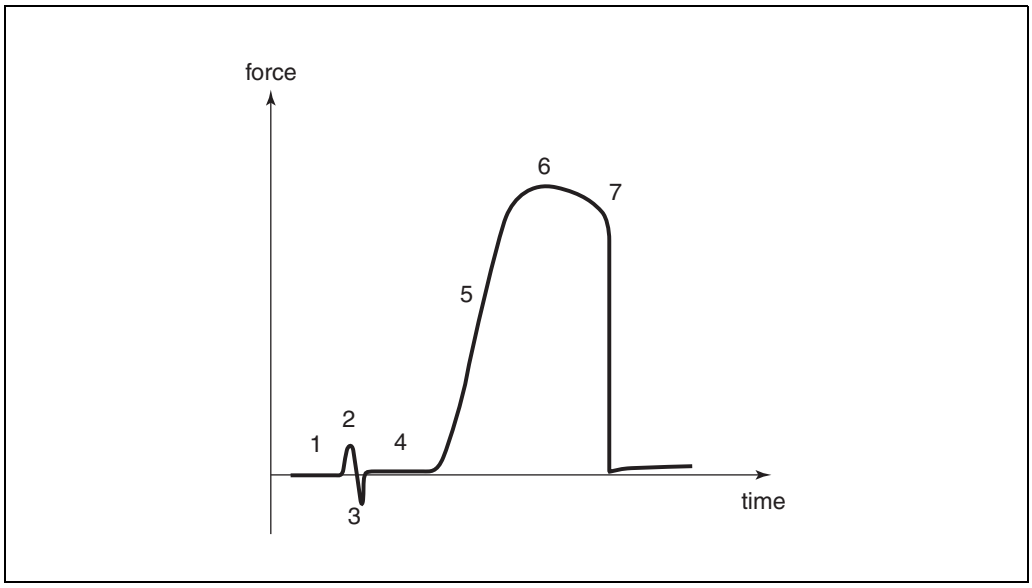

Figure D3.6.2 Force measured as a function of time during an interfacial tension experiment using the du Noüy ring. 1, no wetting; 2, initial wetting; 3, submerging of ring; 4 + 5, pulling of ring from the interface; 6, maximum pull force; 7, ring detachment.

7. Keep lowering the pedestal until a maximum interfacial tension is reached. Record the maximum value of the interfacial tension.

   At this point, a meniscus will be fully formed and the volume of the liquid pulled up by the ring is at its maximum. Subsequent lowering of the pedestal will cause the ring to detach from the surface and the surface tension will immediately decrease to zero.

   The maximum surface tension recorded is proportional to the force needed to remove the ring from the liquid interface and is directly proportional to the surface tension of the liquid. Figure D3.6.2 shows a plot of the recorded force as a function of the experimental time. Modern tensiometers that are connected to a computer not only record the entire experiment but also automatically compute the surface tension.

BASIC PROTOCOL 2

DYNAMIC INTERFACIAL TENSION DETERMINATION BY THE DROP VOLUME TECHNIQUE

A schematic diagram of the drop volume tensiometer (DVT) is shown in Figure D3.6.3. The setup consists of a syringe that contains the sample solution. The plunger of the syringe can be automatically advanced using a step motor controller in order to form droplets at the tip of the syringe with a specific predetermined volume. If drops exceed a critical volume, they will detach from the tip of the syringe due to gravitational force. The critical volume is directly related to the surface tension, which opposes the detachment. The syringe is usually mounted in a temperature-controlled block on top of a glass cuvette that is equipped with a light-emitting diode (LED) detector system to record the drop detachment time. When a droplet detaches from the tip of the capillary, the light beam between the light source and the detector will be briefly interrupted, causing a signal to be transmitted to the input/output interface. The drop detachment time can be used to obtain information about the age of the interface.

For interfacial tension measurements, the cuvette can be filled with a second liquid. Two setups are possible: a top-to-bottom setup and an inverted bottom-to-top setup. In the top-to-bottom setup, the syringe is filled with the higher-density fluid and the cuvette contains the lower-density fluid. The drop subsequently detaches in the direction of the gravitational field. In the inverted setup, a special U-shaped needle is required. Using the

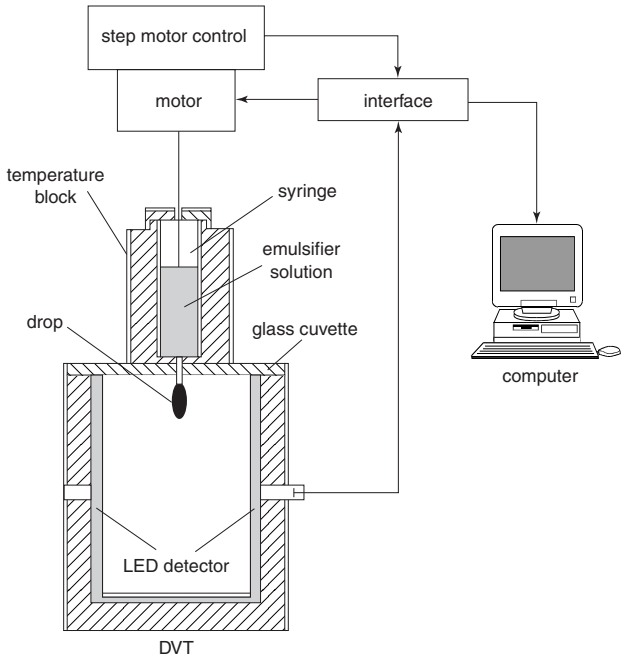

Figure D3.6.3 Schematic diagram of a drop volume technique (DVT) tensiometer.

U-shaped needle, a drop of the less-dense fluid can be formed within the higher-density fluid. The drop will detach opposite to the direction of the gravitational field.

It has recently been shown that the inverted setup is better suited to measurement of the adsorption kinetics of protein samples at, e.g., the oil/water interface since it prevents reservoir depletion. Reservoir depletion can occur if the concentration of surfactant in the solvent phase is low. If the protein is present in the drop-forming phase, then the concentration within the drop itself may decrease during the adsorption process. This in turn would affect the measured rate of adsorption. In this case, it is preferable to form an inverted oil droplet in the protein solvent.

Modern drop volume tensiometers are connected to a computer with sophisticated software that can be used to automatically record the surface tension as a function of the true interfacial age. Adsorption kinetics experiments with the drop volume technique can be conducted using either the constant drop formation method or the quasistatic method (for details, see Commentary). The choice of the dynamic measurement method depends primarily on the time range over which the adsorption kinetics needs to be measured.

While the quasistatic method is quite accurate, it requires a long time to determine a complete adsorption kinetics curve. This is because a new drop has to be formed at the tip of the capillary to determine one single measurement point. For example, if ten dynamic interfacial tension values are to be determined over a period of 30 min, ~180 min will be required to conduct the entire measurement. On the other hand, the constant drop formation method is often limited because a large number of droplets have to be formed without interruption, which may rapidly empty the syringe. Furthermore, the critical volume required to cause a detachment of droplets depends on the density difference between the phases. If the density difference decreases, the critical volume will subsequently increase, which may exacerbate the problem of not having enough sample liquid for a complete run.

Materials

~1 mM chromic acid
Ultrapure water (for calibration)
Sample solution (surface-active liquid)
Drop volume tensiometer (DVT; Figure D3.6.3)

1. Clean the cuvette, syringe, and in particular the syringe tip (made of stainless steel) of a drop volume tensiometer with diluted chromic acid (~1 mM). Rinse extensively with ultrapure water to completely remove any remaining acid.
2. Fill glass cuvette with primary fluid (lower density fluid for regular setup or higher density fluid for inverted setup) and set in cuvette holder.

   For a liquid/gas interface, the primary "fluid" is simply air and the cuvette will therefore remain empty. In this case, extensive cleaning of the cuvette is not required.
3. Fill syringe with the secondary fluid (higher density fluid for regular setup or lower density fluid for inverted setup).
4. Insert cuvette and syringe in syringe/cuvette holder (temperature block).
5. Position syringe for subsequent measurement.

   Usually, the syringe simply needs to be lowered until the tip is submerged in the primary fluid. Note that in some drop volume tensiometers, the final resting position of the holder may need to be adjusted manually. The drop formed at the tip of the syringe must be located just above the LED detector so that the detachment time can be determined accurately.
6. Start the measurement program on the computer.

   A number of physical property data are required for correct calculation of the interfacial tension, e.g., the densities of the primary and secondary fluids at the selected temperature. These should be obtained either from the literature or through preliminary experiments.

ALTERNATE PROTOCOL 3

DYNAMIC INTERFACIAL TENSION DETERMINATION BY THE DROP SHAPE TECHNIQUE

Interfacial tension measurement by drop shape analysis is based on the principle that the shape of a drop formed at the tip of a capillary depends on the interfacial tension. The general setup of the drop shape analysis (DSA) tensiometer is shown in Figure D3.6.4. The device consists of a fluid reservoir that contains the sample fluid. A fluid dispenser is used to form the drop at the tip of a needle, which is suspended in a cuvette that, in the case of interfacial tension measurements, would contain the second, less-dense fluid. A high-speed video camera is used to record up to 60 drop pictures/sec. Each picture is then digitized using a frame grabber that is connected to a computer. The digitized picture is analyzed and the drop profile is extracted. Using the Young-Laplace equation (see Commentary), the interfacial tension is obtained via nonlinear curve fitting of theoretically calculated drop profiles with measured drop profiles. The procedure is performed essentially as described in Basic Protocol 2. The drop shape analyzer is typically supplied with an appropriate syringe, but the cuvette must often be purchased separately. A large-volume glass or quartz cuvette is generally recommended.

While the measurement protocol is fairly simple, there are a number of important factors that need to be considered when using the drop shape analysis method. First, a sufficient visual contrast between the drop and the surrounding liquid is required to be able to extract the drop profile. If the external phase is slightly turbid, or if the refractive indices of the two phases match each other, it may be difficult or even impossible to extract the drop profile. If possible, the more turbid phase should be chosen as the internal drop-forming

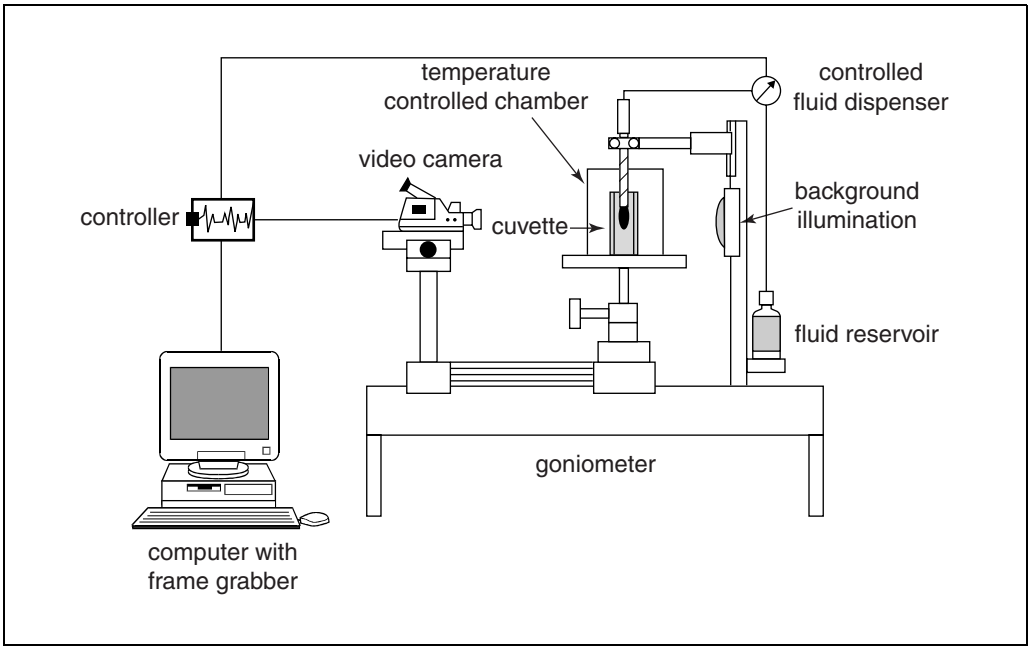

Figure D3.6.4 Schematic diagram of a drop shape analysis (DSA) tensiometer.

phase. Prior to each measurement, the sharpness of the picture should always be double checked and the camera adjusted, if necessary. Modern software is often equipped with a so-called focusing assistant that will allow the operator to adjust the focus. A correct fit of the Young-Laplace equation to the drop profile also requires precise knowledge of the density of the two phases. If the DSA tensiometer is used on a daily basis, the purchase of an automatic densitometer (with an accuracy of at least ±0.002 g/liter) is recommended. The precise density of the sample should be measured prior to each interfacial tension experiment. Additional problems with drop profile extraction may result from insufficient background illumination or extensive reflections on the drop due to illumination in the room. In this case, the instrument can be placed in a darkened room, which will eliminate reflections and increase contrast. Finally, the room temperature should be kept constant to ensure that results do not fluctuate and are consistent.

COMMENTARY

Background Information

An almost overwhelmingly large number of different techniques for measuring dynamic and static interfacial tension at liquid interfaces is available. Since many of the commercially available instruments are fairly expensive to purchase (see Internet Resources), the appropriate selection of a suitable technique for the desired application is essential. Dukhin et al. (1995) provides a comprehensive overview of currently available measurement methods (also see Table D3.6.1). An important aspect to consider is the time range over which the adsorption kinetics of surface-active substances can be measured (Fig. D3.6.5). For applications in which small surfactant molecules are primarily used, the maximum bubble pressure (MBP) method is ideally suited, since it is the only method that allows surface-tension measurements in the millisecond range. However, the method only allows the determination of surface tension and is thus fairly limited. Further information is found in Miller et al. (1992) and Dukhin et al. (1995). For applications in which surface-active substances have relatively low adsorption rates, as is the case in protein and lipid chemistry, the drop shape analysis technique may be suitable. An additional criterion that should be considered when selecting an instrument is whether there is a need to measure interfacial tension at elevated temperatures. Not all instruments are capable of conducting measurements above room temperature (Table D3.6.1).

The following sections briefly discuss the theoretical basis of the measurement methods

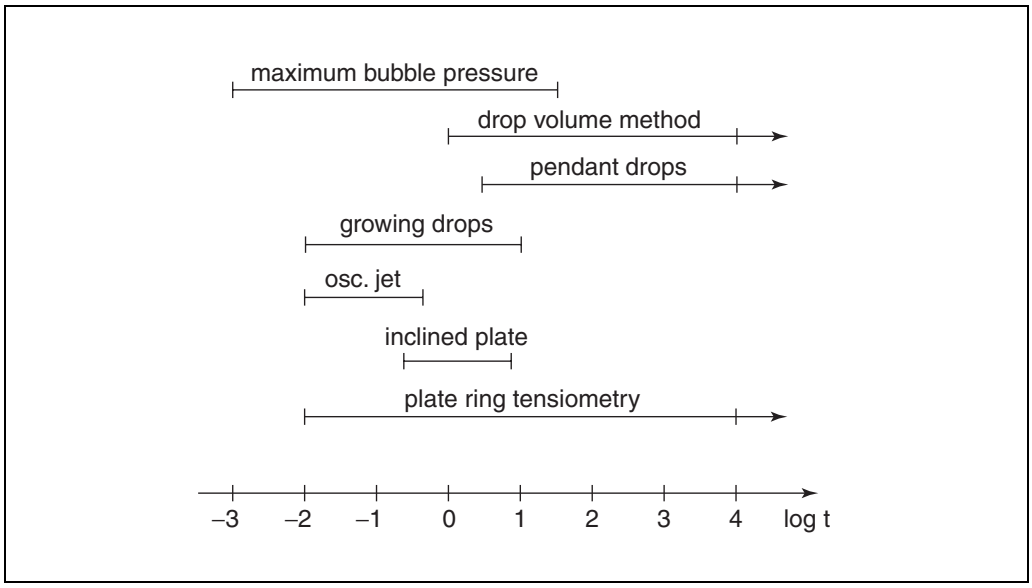

Figure D3.6.5 Overview of time-resolving capabilities of various interfacial tension measurement techniques. Also see Table D3.6.1. Adapted from Dukhin et al. (1995) with permission from Elsevier Science.

that are described in this unit. A more detailed description can be found in Dukhin et al. (1995) and Miller et al. (1992).

du Noüy ring method

Lecomte du Noüy (1919) stated that there were large discrepancies in interfactial tension results obtained with previously available methods, which illustrated the need to develop better measurement methods. He therefore developed a new method in which a platinum ring was lowered into a liquid, and the pulling force of the meniscus on the ring was measured. In the original experimental design, du Noüy directly measured the force, *F*, required to pull a platinum ring from the interface, making the method dependent upoon geometry and thus the device used. Harkins and Jordan (1930) published the theoretical foundation that showed that the method can also be used as an absolute interfacial tension measurement. Fig-

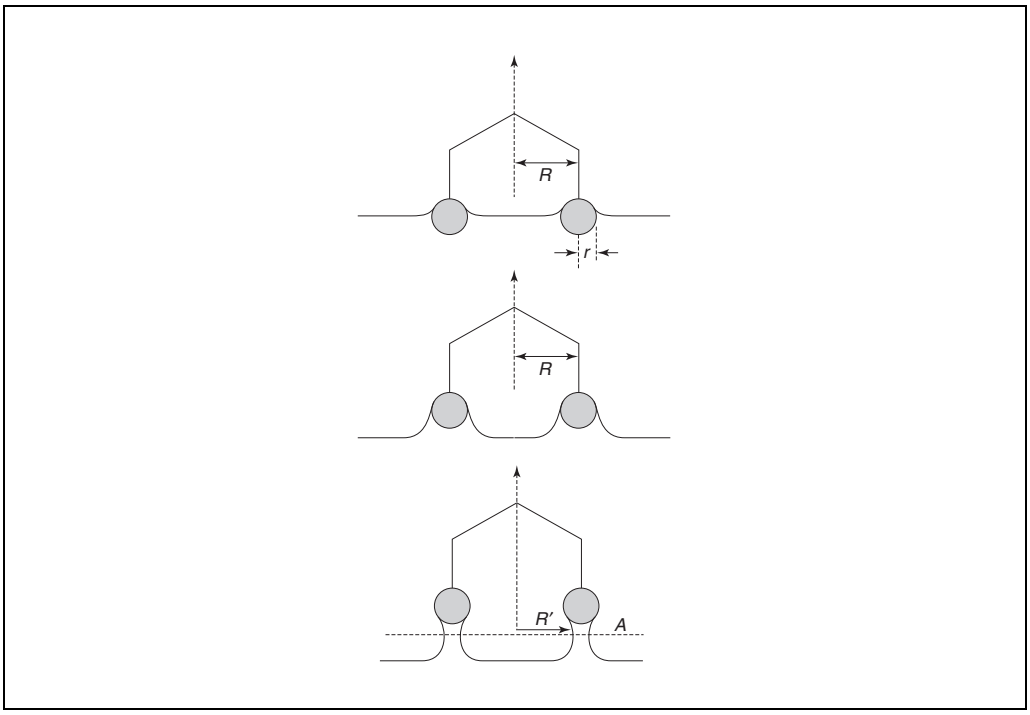

Figure D3.6.6 Principle of du Noüy ring tensiometry.

ure D3.6.6 shows the principle of the method. The total force, F_t, acting on the ring is given as:

$$F_t = F_r + 4\pi R\gamma$$

Equation D3.6.1

where R is the radius of the ring, F_r is the weight of the ring, and γ is the interfacial tension. The authors also determined that a correction factor, f, was required to account for the fact that the inside and outside surface areas of the ring (where the pulling force acts) are not equal (Harkins and Jordan, 1930). The correction factor f is a function of the radius of the ring, R, and the radius of the wire, r. Zuidema and Waters (1941) later expanded this correction factor to account for the situation in which the interfacial tension of two phases with similar densities (e.g., hydrocarbon and water) is measured.

Wilhelmy plate method

Wilhelmy (1863) suggested that the interfacial tension of liquids could be determined by measuring the maximum force required to pull a glass plate vertically from the interface. In his experiment, he was careful to ensure that the glass plate was extremely clean so that the angle of contact (ϕ) was relatively small or close to zero. The force, F, exerted on the plate raises the meniscus of the fluid above the level of the flat surface as shown in Figure D3.6.7. The mass of the liquid that is elevated above the fluid interface increases to some maximum value as F increases. Once the meniscus is fully formed, the force acting on the plate is equal to the interfacial tension multiplied by the circumference of the plate, as long as the following conditions are met: (1) the wetting angle (ϕ) is close to zero, that is, the plate supporting the liquid is completely wet; (2) buoyancy effects can be neglected, that is, the lower edge of the plate is at or above the level of the bulk fluid interface; and (3) the sides of the plate are parallel and the plate is perpendicular to the fluid interface.

The Wilhelmy plate method was in later years improved by replacing the glass plate with a slightly roughened platinum plate. An advantage of the Wilhelmy method in contrast to the du Noüy method is that it does not require a correction factor. Figure D3.6.7 shows a representative illustration of the meniscus attached to the plate. The force acting on the plate is simply the sum of interfacial tension and the buoyancy effect of a column of liquid pulled up under the plate (assuming that the wetting angle is zero):

$$F_t = F_b + p\gamma$$

Equation D3.6.2

where F_t is the total force and p is the circumference of the plate. A buoyancy force, F_b, needs to be added or subtracted depending on whether the plate is above or below the interface of the fluid:

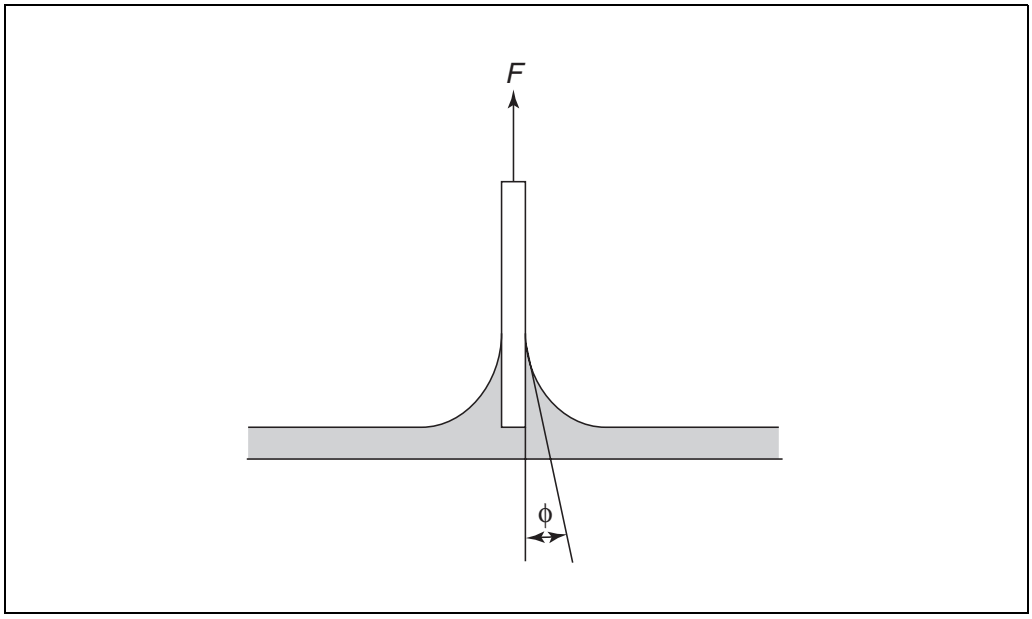

Figure D3.6.7 Principle of the Wilhelmy plate method.

$$F_b = \Delta\rho g h A$$

Equation D3.6.3

where $\Delta\rho$ is the density difference between the two phases, g is gravitational acceleration, h is the height above or below the interface, and A is the cross-sectional area of the plate.

Drop volume technique

The theoretical foundation of the drop volume technique (DVT) was developed by Lohnstein (1908, 1913). Originally, this method was only intended to determine static interfacial tension values. Over the past 20 years, the technique has received increasing attention because of its extended ability to determine dynamic interfacial tension. DVT is suitable for both liquid/liquid and liquid/gas systems. Adsorption kinetics of surface-active substances at liquid/liquid or liquid/gas interfaces can be determined between 0.1 sec and several hours (see Fig. D3.6.5).

The principle of this technique is based on the measurement of the volume of a drop that is formed at the tip of a syringe. The drop is retained at the tip of the syringe as long as the interfacial tensile force is larger than the opposing gravitational force due to the mass of the droplet. When the drop has grown large enough, it will detach from the tip of the syringe at which point the two opposing forces, tensile and gravitational force, are exactly equal. Thus, the interfacial tension can be calculated if the precise volume of the drop at the moment of detachment is known.

Figure D3.6.8 illustrates the different phases of drop development and detachment during the measurement. As shown in Figure D3.6.8, the drop does not detach at the exact tip of the capillary. Instead a "neck" is formed at which the liquid meniscus will eventually be disrupted. The radius of the neck is smaller than the radius of the capillary, r_{cap}. A force balance on the drop yields:

$$2\pi r_{cap}\phi\gamma = V\Delta\rho g$$

Equation D3.6.4

where V is the volume of the drop, $\Delta\rho$ the density difference between the two phases, g the gravitational acceleration, and ϕ an empirically derived correction factor that takes the neck formation into account. Experiments by Harkins and Brown (1919) have shown that ϕ is a function of the capillary radius and the drop volume:

$$\phi = f\left(\frac{r_{cap}}{\sqrt[3]{V}}\right)$$

Equation D3.6.5

As described below, two different measurement variations exist to determine adsorption kinetics of surface-active substances: continuous drop formation and quasistatic measurements.

Continuous drop formation

The adsorption kinetics of surface-active substances at liquid/liquid and liquid/gas inter-

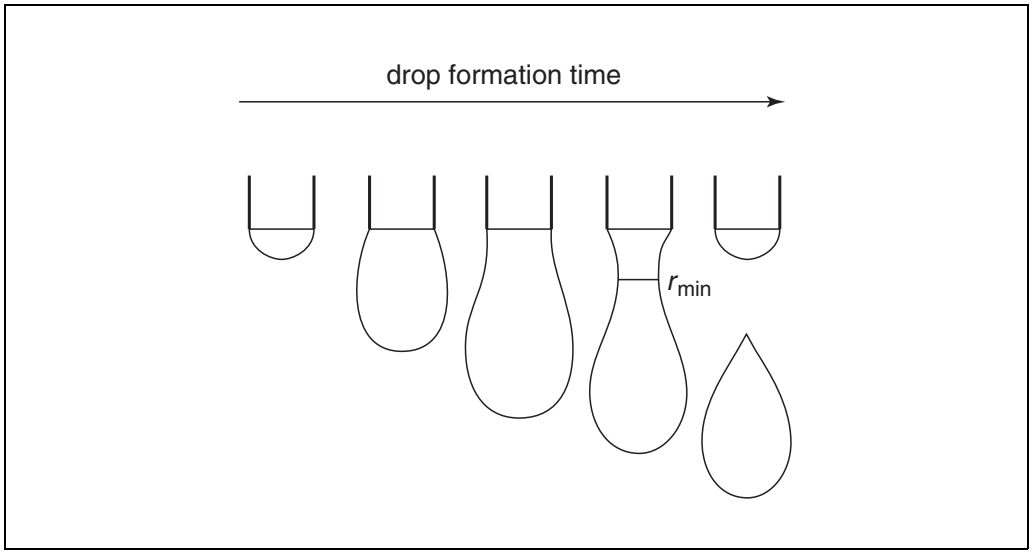

Figure D3.6.8 Drop growth at the tip of a capillary and subsequent drop detachment during adsorption kinetics measurements using the drop volume technique (DVT).

faces can be determined with the drop volume method through continuous drop formation at the tip of the capillary by advancing the syringe plunger with a specific rate using a step-controlled motor. During the growth of the droplet, surfactant molecules will migrate from within the drop-forming phase that contains the emulsifier and adsorb at the interface of the droplet, subsequently decreasing the interfacial tension. The drop will detach from the tip of the syringe once it reaches its critical volume. This critical volume is smaller than the critical volume of a drop that does not contain any surface-active material. The time between drop formation and detachment is therefore related to the adsorption kinetics. It contains explicit information about the age of the interface. By varying the flow rate, the age of the interface can consequently be varied between several seconds and several minutes.

The continuous formation of drops, however, can lead to substantial errors in obtained adsorption kinetic data. For short drop formation times, hydrodynamic effects have to be taken into account. At large flow rates, the measured drop volume at the moment of detachment must be corrected. This is because a finite time is required for the drop meniscus to be disrupted and the drop to detach. Even though the volume has already reached its critical value, fluid may still flow from the reservoir into the drop. The volume of the drop is thus larger than its measured value, which leads to larger calculated interfacial tension values. The shorter the drop formation time is, the larger the error will be. Kloubek et al. (1976) were the first to quantify this effect by introducing a corrected critical drop volume, V_c:

$$V_c = Vt - \frac{k}{t}$$

Equation D3.6.6

where k is a regression coefficient that depends on the density difference between the two phases, the radius of the capillary, and the surface tension. Additional empirical corrections were given by Jho and Burke (1983):

$$V_c = Vt - kt^{\frac{3}{4}}$$

Equation D3.6.7

The problem becomes even more complex at high flow rates because of fluctuations in the measured volumes of the droplets. Fainerman and Miller illustrated that this irregular behavior may be based on the formation of capillary waves that lead to stable bifurcations that complicate the pattern of drop volume oscillations (Fainerman and Miller, 1995; Miller et al., 1998). Since this effect is based on hydrodynamic effects, the viscosity of the drop fluid and the radius of the capillary can affect the end result. As viscosity and capillary radius increase, higher apparent interfacial tension values are obtained. While correction functions have been developed that yield realistic interfacial tension data, it should be verified that the software that operates the DVT incorporates the latest physical models.

To determine the adsorption kinetics, the effective age of the drop interface must be calculated. However, experimental data yield only interfacial tension values as a function of drop formation time. To determine the true age of the interface, both the fluid flow within the droplet and the dilation of the droplet interface must be interpreted using appropriate models. Miller et al. (1992) showed that the drop formation time, t_{drop}, can be converted into the effective age of the drop interface:

$$\tau_{eff} = \frac{t_{drop}}{3}$$

Equation D3.6.8

Quasistatic measurements

Initially, a drop with a specific volume is very rapidly formed at the tip of the syringe. The drop volume is slightly smaller than the critical volume that corresponds to the equilibrium interfacial tension at which the drop would ordinarily detach. The drop will therefore remain attached to the tip surface. As surface-active material adsorbs at the liquid interface, the interfacial tension decreases and the drop will eventually detach. The time required between drop formation and drop detachment is the so-called drop detachment time. If the time required to form the drop is small compared to the drop detachment time, then the drop detachment time can be set equal to the effective age of the interface. Gradually, reducing the drop volume will increase the time required for the drop to detach. The drop detachment time and thus the age of the interface can be varied between ~10 sec and 30 min.

Drop shape analysis

This technique is based on the determination of the shape of a pendant drop that is formed at the tip of a capillary. The classical form of the Young and Laplace equation relates the pressure drop (Δp) across an interface at a given point to the two principal radii of curvature, r_1 and r_2, and the interfacial tension (Freud and Harkins, 1929):

$$\Delta p = \gamma\left(\frac{1}{r_1} + \frac{1}{r_2}\right)$$

Equation D3.6.9

For a pendant drop that is in mechanical equilibrium with a surrounding fluid, the balance between the capillary and the buoyancy forces can be expressed as:

$$\Delta p = \gamma\left(\frac{1}{r_1} + \frac{1}{r_2}\right) = \frac{2\gamma}{r_0} + \Delta\rho g z$$

Equation D3.6.10

where r_0 is the radius of the curvature at the origin of the *x-z* coordinate systems, g is the gravitational acceleration, and $\Delta\rho$ is the density difference between the drop phase and the surrounding liquid phase. The basic profile of an axisymmetric drop can then be calculated using the following differential equations:

$$\frac{d\phi}{ds} = 2 - \beta y - \frac{\sin\phi}{x}$$

$$\frac{dx}{ds} = \cos\phi$$

$$\frac{dy}{ds} = \sin\phi$$

Equation D3.6.11

where *x, y,* and *z* are dimensionless variables based on the horizontal and vertical coordinates and the arc length, *s*, of the profile measured from the drop apex by the radius of the curvature in the drop apex, r_0. The parameter β is an indicator for the drop shape and is given as:

$$\beta = \frac{\Delta\rho g r_0^2}{\gamma}$$

Equation D3.6.12

A set of experimental drop profiles is usually nonlinearly fitted to numerically generated drop profiles that are based on the solution of the Laplace equation. It has also been shown that the interfacial tension can be calculated from two characteristic diameters, d_e and d_s. Girault et al. (1984) derived the following relationship:

$$\frac{\Delta\rho g r_0^2}{\gamma} = 0.02664 + 0.62945\left(\frac{d_s}{d_e}\right)^2$$

Equation D3.6.13

The radius of the curvature, r_0, can be obtained from the following relationship:

$$\frac{d_e}{2r_0} = 0.9987 + 0.1971\beta - 0.0734\beta^2 + 0.34708\beta^3$$

Equation D3.6.14

which is valid for $0.1 < \beta < 0.5$. Figure D3.6.9 shows an overview of the drop coordinates and parameters.

Critical Parameters

Interfacial tension is highly sensitive to even a small concentration of contaminants. It is therefore essential to keep samples and equipment as clean as possible. Both the plate or ring and all sample containers should be extensively rinsed with ultrapure water. Using disposable pipets is recommended. If interfacial tension measurements are a standard method that will be conducted on a daily basis, a water purification system that produces freshly deionized water should be installed in the laboratory. The ultrapure water should be transported or stored in containers made of perfluorinated polymers such as Teflon or FEP. Polypropylene, which is considerably less expensive, can be used as an alternative since it becomes cleaner with extended use. The use of glass should be avoided because, although glass may not have organic contaminations, there may be an appreciable dissolution of silicate (up to 10 mM). To verify the pH of water, a specifically designed pH meter appropriate for use with ultrapure water must be used. Note that ultrapure water that has been properly equilibrated with air should have a pH of 5.5. A quick test to check the accuracy of interfacial tension measurements is to determine the surface tension of ultrapure water, which should be 72.4 mN/m at 20°C. A properly calibrated standard tensiometer is expected to have an accuracy of better than ±0.1 mN/m.

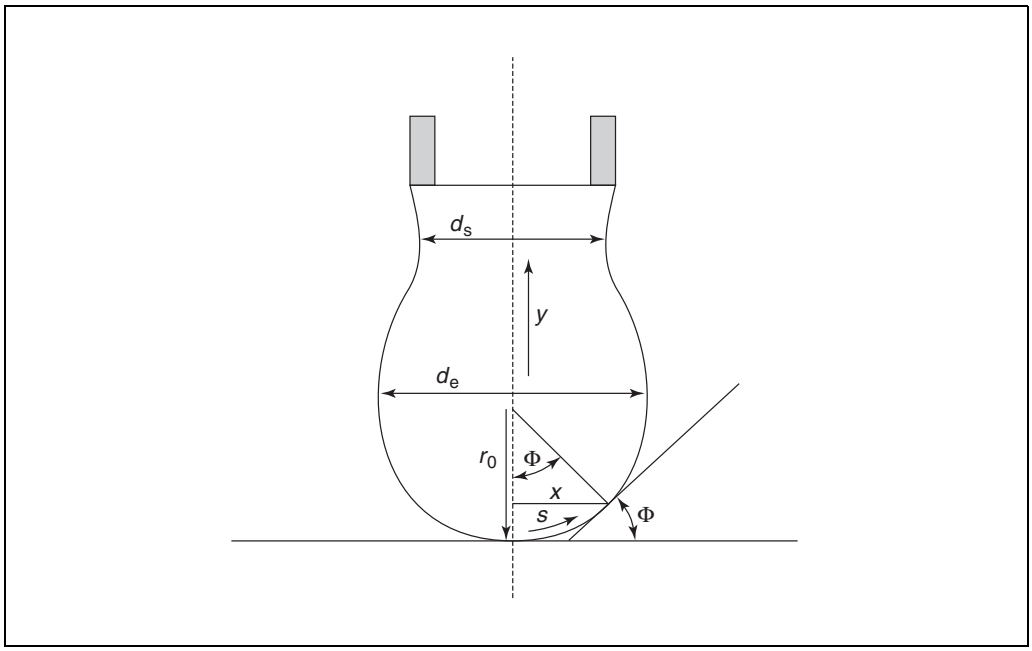

Figure D3.6.9 Characteristic geometric parameters of a drop formed at the tip of a capillary. Adapted from Dukhin et al. (1995) with permission from Elsevier Science.

Anticipated Results

The Wilhelmy plate and du Noüy ring methods provide a single value for surface tension of a given surfactant at a given concentration. Examples are provided in *UNIT D3.5* (see Figure D3.5.5). Examples of dynamic surface tension values are also provided in *UNIT D3.5*.

The drop volume and drop shape techniques provide multiple interfacial tension values as a function of the interfacial age for a given surfactant at a given concentration at a specific temperature. Examples are provided in *UNIT D3.5* (see Fig. D3.5.6).

Time Considerations

The time required to conduct an interfacial tension experiment depends largely on the properties of the surfactants and less on the chosen measurement method. A notable exception is the drop volume technique, which, due to the measurement principle, requires substantially more time than the drop shape analysis method. Regardless of the method used, 1 day or more may be required to accurately determine, e.g., the adsorption isotherm (*UNIT D3.5*) of a protein. This is because, at low protein concentrations, it can take several hours to reach full equilibrium between proteins in the bulk phase and those at the surface due to structural rearrangement processes. This is especially important for static interfacial tension measurements (see Basic Protocol 1 and Alternate Protocols 1 and 2). If the interfacial tension is measured before the exchange of molecules between interface and bulk phase has reached equilibrium, the interfacial tension may continue to decrease. In the case of the du Noüy ring, this can substantially increase the time requirements, since the measurement will have to be repeated several times at increasing time intervals to ensure that the interfacial tension values are truly static. On the other hand, the rate of adsorption of concentrated protein solutions at the oil-water interface can be very high. As a result, it may take <10 min for full equilibrium to be reached. In this case, proper sample preparation and cleaning may be more time consuming than the experiment itself.

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Key Reference

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An excellent comprehensive review of all theoretical and practical aspects of dynamic and static interfacial tension measurements written by the most prolific authors in the field of protein adsorption. Contains a wealth of additional references that the interested reader may consult to gain additional understanding of the field of research.

Internet Resources

http://www.lauda.de/e/home-e.htm

The LAUDA measuring instruments program covers systems for measuring the surface and interfacial tension of liquids. Available devices include ring plate tensiometers (automated and nonautomated), drop volume tensiometers, bubble pressure tensiometers, and film balances.

http://www.kruss.de/krussdframed_e.html

Provides measuring techniques of contact angle, surface tension, interfacial tension, and bubble pressure. Suitable methods for both static and dynamic interfacial tension of liquids include du Noüy ring, Wilhelmy plate, spinning drop, pendant drop, bubble pressure, and drop volume techniques. Methods for solids include sessile drop, dynamic Wilhelmy, single fiber, and powder contact angle techniques.

http://www.rheosci.com/products/surface/

Specializes in rheological properties of materials but has recently expanded its product line to include surface characterization mechanisms. Provides dynamic contact angle fiber tensiometer, dynamic contact angle tensiometer, and contact angle tensiometer.

Contributed by Jochen Weiss
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Colorimetric Quantification of Carbohydrates

In many projects involving a transformation or isolation of carbohydrates, it is important to quantify the amount of product obtained. It is sometimes difficult to measure the content of a specific component in a mixture. The process can be laborious with instrumentation such as HPLC or GC, especially if the sample contains major impurities of different natures. Fortunately, knowledge of the properties of the desired product can suggest a specific method of analysis, several of which involve colorimetric analysis. With colorimetric analyses, a selective chemical transformation will produce color. The absorbance or transmittance of the colored sample is directly correlated with the presence of a certain functional group or product in the mixture. Various reactions that produce color are known to be selective for certain types of carbohydrates.

Three spectroscopic procedures to quantify sugars are described in this unit. All of the methods use the same principles but are selective for the quantification of different sugars. In all cases, the absorbance of a colored sample, resulting from a simple specific chemical transformation, is measured. Transmittance can also be used for measurement. Quantification of the amount of sugar using colorimetry can be an accurate and convenient technique. Among several methods available, the phenol-sulfuric acid assay (see Basic Protocol) is most frequently used. Unfortunately, the procedure is unsatisfactory when quantifying certain deoxy- and amino-sugar derivatives. In such cases, the Morgan-Elson assay can be performed (see Alternate Protocol 1). The Somogyi-Nelson method (see Alternate Protocol 2), can be specific to reducing sugars.

For any sugar determination, the specific quantification method chosen should selectively distinguish the different carbohydrates present in a same mixture.

CAUTION: Wear appropriate protective clothing, i.e., gloves and protective glasses. It is preferable to perform most manipulations in a fume hood, especially when handling strong acids and chemical reagents. Refer to the MSDS of individual compounds for more information.

IMPORTANT NOTE: All samples must be centrifuged or allowed to precipitate before any measurement to remove any insoluble material.

NOTE: All pipets and reagent dispensers must be well calibrated for accuracy.

DETERMINATION OF REDUCING AND NONREDUCING SUGARS USING THE PHENOL-SULFURIC ACID ASSAY

BASIC PROTOCOL

It is sometimes necessary to quantify the amount of sugar in a certain medium. Whether the sugar is in the presence of various salts or protein residues, or attached to a polymer, the phenol-sulfuric acid assay can be performed. With the exception of certain deoxy-sugars, the method is very general, and can be applied to reducing and nonreducing sugars and to many classes of carbohydrates including oligosaccharides. Determination of sugars using phenol-sulfuric acid is based on the absorbance at 490 nm of a colored aromatic complex formed between phenol and the carbohydrate. The amount of sugar present is determined by comparison with a calibration curve using a spectrophotometer. Under the proper conditions, the phenol-sulfuric acid method is accurate to ±2%. If a spectrophotometer is not available, the method can be performed qualitatively by direct visual comparison with colored samples of known concentration.

Contributed by Eric Fournier

Current Protocols in Food Analytical Chemistry (2001) E1.1.1-E1.1.8

Materials

4% phenol: 40 g phenol (reagent grade) in 1 liter distilled water, store up to 6 months at room temperature
96% sulfuric acid (reagent grade)
1 mg/ml sugar standards (reagent grade) stored in sealed tubes

Sample
10-ml test tubes
Spectrophotometer
500-µl and 2.5-ml bottle-top reagent dispensers
5 to 200-µl and 200 to 1000-µl pipets

IMPORTANT NOTE: Cellulose can interfere with the experiment; all contact with paper dust must be avoided.

1. Wash the 10-ml test tubes with distilled water. Set the spectrophotometer at 490 nm. Allow the lamp of the spectrophotometer to warm up and zero the instrument with a blank solution of 500 µl 4% phenol and 2.5 ml of 96% sulfuric acid.

2a. *For samples containing a single sugar unit (monosaccharides or simple polysaccharides):* Prepare calibration sugar standards using 1 mg/ml sugar standard solutions, of the same sugar present in the test sample, in distilled water. Transfer aliquots to 10 different, dry 10-ml tubes in 5-µl increments ranging from 5 to 50 µl, with an accurate pipet.

When only one sugar unit is present, only that specific sugar should be used. As an example, maltose is a polysaccharide composed of two glucose units. To get accurate results in the determination of maltose, use commercially available maltose or glucose.

2b. *For samples containing different sugar units (monosaccharide mixtures or complex polysaccharides):* Mix stoichiometric amounts of all of the component sugar units present in the sample and prepare a 1 mg/ml (total) solution in distilled water. Prepare calibration sugar standards by transferring aliquots to 10 different 10-ml tubes in 5 µl increments from 5 to 50 µl with an accurate pipet.

When different sugar units are present, all the sugars units in the standards should be mixed in the same proportion as in the sample. As an example, lactose is a disaccharide composed of a galactose and glucose unit. To get accurate results in the determination of lactose, use commercially available lactose or an equivalent stoichiometric mixture of galactose and glucose .

3. Transfer a portion of the sample to be analyzed to a 10-ml test tube; note the volume or weight of the sample taken.

At this point, all the tubes should contain between 5 to 50 µg of sugar, and one should contain an unknown amount of sugar to be determined.

4. To all the tubes, add 500 µl of 4% phenol followed by 2.5 ml 96% sulfuric acid.

All the glycosidic linkages are broken and the colored complex is formed in this step.

In contact with water, sulfuric acid produces some heat.

5. Transfer the solutions from the test tubes to the cuvettes and measure the A_{490} of the sugar standards and unknown solutions.

If the A_{490} is higher than 1.0 OD unit, or higher than all the standards, dilute the sample with distilled water.

6. To calculate the concentration of sugar present in the sample, make a graph plotting A_{490} versus sugar weight (µg) of the sugar calibration standards.

The intercept of the A_{490} of the unknown sample with the calibration line represents the amount (µg) of sugar present in the sample.

7. Calculate the unknown sample concentration using the following equations:

where x is the mass (g) of sugar sample deduced from the graph, mol. wt. represents the

$$\text{concentration (mol/g)} = \frac{x(\text{g})}{\text{Mol. wt (g/mol)} \times \text{weight (g)}}$$

$$\text{percentage of sugar (\% by weight)} = \frac{x(\text{g})}{\text{weight (g)}} \times 100$$

molecular weight of the monosaccharide or polysaccharide in the sample, and w is the weight (g) of the sample (step 3).

Concentrations in µmol/liter can be calculated by replacing w(g) of the equation by v (liters), where v (liters) is the volume in liters (step 3).

DETERMINATION OF REDUCING SUGARS USING THE SOMOGYI-NELSON METHOD

ALTERNATE PROTOCOL 1

If all the sugars present in a certain medium are expected to be reducing sugars, or to determine the content of total reducing sugar, the Somogyi-Nelson method can be performed. This method utilizes the reducing properties of certain types of carbohydrates. Determination of reducing sugar using Somogyi-Nelson is based on the absorbance at 500 nm of a colored complex formed between a copper-oxidized sugar and arsenomolybdate. The amount of carbohydrate present is determined by comparison with a calibration curve using a spectrophotometer. Under the proper conditions, the Somogyi-Nelson method is accurate to ± 0.01 mg for D-glucose, D-galactose, and maltose. The most consistent results are obtained when operations are carried out under inert atmosphere, and when the measured concentrations do not exceed 1 mg/ml. If a spectrophotometer is not available, the method can also be performed qualitatively.

Additional Materials *(also see Basic Protocol)*

Low-alkalinity copper reagent (see recipe)
Arsenomolybdate reagent (see recipe)
Boiling water bath

1. Wash 10-ml test tubes with distilled water. Set the spectrophotometer at 500 nm. Allow the lamp of the spectrophotometer to warm up, and zero the instrument with a prepared blank solution.

   The blank is prepared with 1 ml of distilled water, adding low-alkalinity copper reagent and arsenomolybdate reagent as described in step 4.

2. Prepare sugar standards using a 1 mg/ml solution of the commercially available reducing sugar present in the sample, in distilled water. Transfer aliquots of 5 to 100 µl, or 100 to 600 µl with an accurate pipet to different 10-ml tubes.

3. Transfer a portion of the sample mixture to be analyzed to a 10-ml test tube; note the volume or weight of the sample taken.

   At this point, all test tubes should contain 5 to 100 µg or 0.1 to 0.6 mg of sugars, with one containing an unknown amount of sugar to be determined.

4. To all the tubes, add 1 ml low-alkalinity copper reagent. Heat the tubes in boiling water for 10 min. Add 1 ml of arsenomolybdate reagent to tubes containing ≤0.1 mg sugar or 2 ml arsenomolybdate reagent to tubes containing 0.1 to 0.6 mg sugar.

The color should appear after the addition of 1 or 2 ml of the arsenomolybdate reagent. All volumes should be diluted to 5 ml with distilled water and allowed to stand at least 15 min at room temperature.

5. Transfer the solutions from test tubes to cuvettes. Measure the A_{500} of the sugar standards and unknown solutions.

   If the A_{500} measured is greater than 1.0 OD unit, dilute the samples.

6. To calculate the concentration of sugar present in the sample, make a graph of A_{500} versus weight (mg) of sugar. Calculate the concentration from the intercept (see Basic Protocol, step 7).

ALTERNATE PROTOCOL 2

DETERMINATION OF AMINO SUGAR DERIVATIVES USING THE MORGAN-ELSON METHOD

It is sometimes necessary to measure the amount of *N*-acetyl- or amino-sugars in a certain medium. In the Morgan-Elson method, *N*-acetyl- or amino-sugars are heated in an alkaline solution to form a chromogen, which produces a purple colored compound when reacted with *N,N*-dimethyl-*p*-aminobenzaldehyde in an acid solution (Ehrlich reagent). The amount of sugar present is determined by comparison with a calibration curve using a spectrophotometer at the appropriate wavelength of 530 for amino-sugars, and either 544 or 585 nm for *N*-acetyl-sugars. The Morgan-Elson method is accurate to about ±10%, under proper conditions.

***Additional Materials** (also see Basic Protocol)*

1 mg/ml sugar standards (reagent grade): D-glucosamine, D-galactosamine, or *N*-acetyl-D-glucosamine
2,4-pentadione solution (see recipe)
Ehrlich reagent solution I (see recipe)
Boiling water bath
0.8 M potassium tetraborate
Ehrlich reagent solution II (see recipe)

1. Set the spectrophotometer at 530 nm for the measurement of amino-sugars, and at 544 or 585 nm for the measurement of *N*-acetyl-sugars. Allow the lamp of the spectrophotometer to warm up and zero the instrument with a prepared blank solution.

   The blank is prepared with 1 ml of distilled water, adding the reagents as described in step 4a or 4b.

2. Prepare standards using 1 mg/ml reducing sugar standards composed of solutions of commercially available sugars in distilled water. Transfer aliquots of 5 to 20 µl or 10 to 50 µl with an accurate pipet to different 10-ml tubes.

   More reproducible results are obtained with amounts of 5 to 20 µg for N-acetyl-D-glucosamine and amino-sugars, and 10 to 50 µg for N-acetyl-D-galactosamine.

3. Transfer a portion of the sample to analyze to a 10-ml test tube; note the volume or weight of the sample taken.

4a. *For the measurement of amino-sugars:* To all the test tubes, add 2 ml 2,4-pentadione solution and heat the tubes in boiling water for 1 hr. Add 2 ml of Ehrlich reagent solution I.

   The red color produced is stable for <1 hr.

4b. *For the measurement of N-acetyl-sugars*: To all the test tubes containing sugars, add 1 ml 0.8 M potassium tetraborate solution and heat the tubes in boiling water for 10 min. Cool the tubes in ice water and add 3 ml Ehrlich reagent solution II.

The red color produced is stable for <1 hr.

5. Transfer the solution from the test tubes to the cuvettes and measure the absorbance of the reducing sugar standards and unknown solutions.

If the absorbance of the unknowns are higher than 1.0 OD unit, or higher than all the standards, dilute the samples.

6. To calculate the concentrations of sugar present in the sample, make a graph of the absorbance versus weight mass (mg) of sugar. Calculate the concentration from the intercept (see Basic Protocol 1, step 7).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Arsenomolybdate reagent

Prepare a solution of 25 g ammonium molybdate in 450 ml distilled water. Add, with stirring, 21 ml concentrated sulfuric acid and 25 ml of distilled water containing 3 g disodium hydrogen arsenate heptahydrate. Continue stirring 24 hr at 37°C, then store the solution in a 1 liter glass-stoppered brown bottle up to 6 months at room temperature.

Instead of a brown bottle, a bottle covered with aluminum foil can be used to protect the solution from light.

Ehrlich reagent solution I

530 mg *N*,*N*-dimethyl-*p*-aminobenzaldehyde
20 ml ethanol
15 ml concentrated hydrochloric acid
Store up to 1 month at 0°C

Ehrlich reagent solution II

1 g of *N*,*N*-dimethyl-*p*-aminobenzaldehyde
50 ml glacial acetic acid
1.5 ml 10 N hydrochloric acid
Store up to 1 month at 0°C

Low-alkalinity copper reagent

Prepare a solution of 12 g sodium potassium tartrate and 24 g anhydrous sodium carbonate in 250 ml distilled water. Add a solution of 4 g copper sulfate pentahydrate and 16 g sodium hydrogen carbonate in 200 ml distilled water. Separately, prepare a solution of 180 g anhydrous sodium sulfate in 500 ml of boiling distilled water. Combine the two solutions in a volumetric flask and dilute the final solution to 1 liter. Store up to 1 year at room temperature.

Anhydrous sodium sulfate is added to the combined alkaline copper reagent to suppress back-oxidation by air.

2,4-Pentadione solution

Add 2 ml 2,4-pentadione to a 50 ml solution of 1 M sodium carbonate.

The solution is stable, but should preferably be prepared fresh each time.

COMMENTARY

Colorimetric determination of sugars has been recognized for a long time. The most common method is that employing phenol and sulfuric acid (Dubois, 1951; Koch, 1951), which was developed particularly for the determination of lactose in milk products (Barnett, 1957). This assay is currently used because it is simple and the material needed is readily available. Earlier, Nelson (1944) reported a colorimetric modification of a micro copper titrimetric method developed by Somogyi (1937). In the original paper by Somogyi (1926), a component was erroneously printed as sodium carbonate instead of sodium sulfate (Somogyi, 1952). A major objection to the Somogyi assay was the back-oxidation by air. Sodium sulfate is now added to suppress back-oxidation. Thereafter, the method first introduced in 1926 was modified several times by different groups. Consequently, the variation of the Somogyi-Nelson method in use today has been commonly used only since 1954 (Wager, 1954). Despite newer, similar techniques (Cheronis, 1957), the Somogyi-Nelson method was long used for the determination of blood sugar level.

To measure amino-sugar derivatives from different sources, a method developed by Morgan and Elson is currently used (Elson and Morgan, 1933; Cornforth and Firth, 1958). The measurement of amino-sugars is occasionally performed indirectly by condensation with acetylacetone, which subsequently reacts with the Ehrlich reagent. In this case, the method is commonly called Elson-Morgan (Elson and Morgan, 1933). When the *N*-acetyl-sugars react directly with Ehrlich reagent the method is called Morgan-Elson (Morgan and Elson, 1934). The method in use today (Alternate Protocol 2), includes some modifications (Aminoff, 1952).

Most colorimetric determination methods have not been modified since 1957 or earlier. Therefore, colorimetric analyses described in recent literature refer to the early protocols (Whistler, 1962; Birch, 1985; Beauchemin, 1995). Colorimetric carbohydrate analysis for characterization purposes is still a valuable tool as documented in recent chemistry publications (Nilsson, 1997).

Critical Parameters and Troubleshooting

Spectophotometric parameters

Disposable plastic cuvettes are more convenient to use in spectrophotometric detection; however, plastic melts after extended exposure to strong acids. In addition, for more linearity and accuracy in the measurements, many important parameters must be considered. It is advantageous to use cuvettes with path lengths of (0.01 to 0.5 cm), because they use a smaller volume of sample and often give more reproducible results.

Absorbances measured at higher than 1.0 OD unit can affect the linearity of the absorbance versus concentration, and therefore the samples must be diluted if the absorbance measured is higher then 1.0 OD unit or higher then the prepared standards. Sometimes, insoluble residues will also affect the experiment. The samples must be centrifuged or allowed to precipitate before any measurement.

Colorimetric detection

When a determination is critical, it is often necessary to do two or more different colorimetric tests in parallel. A substance present in the sample could interfere with one test while not affecting others. In most cases, interference can be controlled. Interference can be detected by adding a known amount of sugar to a sample solution to be measured. The amount of sugar will differ from what is expected if there is interference. In addition, a very colored sample can interfere with the results obtained. In the case of colored samples, subtract the absorbance of the samples dissolved in water or buffer alone from the value obtained after addition of the colorimetric reagents.

In the phenol-sulfuric acid assay, the major interference known is caused by cellulose; all contact with paper dust must therefore be avoided (Dubois, 1979). Otherwise, there are no reports of significantly common interference.

In the Somogyi-Nelson assay, all substances with reducing or oxidative properties might cause interference. The most important factor to consider is the back-oxidation by air. To control air oxidation, sodium sulfate is added during the process. In addition, citric acid has been reported to cause interference in the estimation of reducing sugars (Paleg, 1959).

In the Morgan-Elson assay, amino acids alone do not interfere, but could alter the result in synergy with other type of sugars. In all colorimetric tests, the quality and stability of the reagents is crucial in order to obtain reproducible results. To eliminate the effect of poor-quality reagents, results should always be validated by measuring a sample of known concentration. If the results differ, new reagents should be prepared. Depending, on the assay, color could vary with time.

Anticipated Results

The three colorimetric analyses described in this unit should give consistent results, provided there is no major interference and the sample concentrations do not exceed the concentrations of the standards. In the appropriate range, all assays described in this unit are accurate between 2% and 10%. The spectroscopic detection in this unit allows the measurement of the lowest standard concentration, i.e., usually 5 μg or more of sugar.

Time Considerations

The reagents are usually prepared in large quantity and are stable for a relatively long period. Therefore, once the reagents are prepared, 1 to 2 hr is appropriate for all manipulations. The phenol-sulfuric acid assay (see Basic Protocol), requires an additional ~30 min for the preparation of the reagents. A total of ~2 hr is necessary from the beginning of the experiments to the obtaining the results.

The Somogyi-Nelson assay (see Alternate Protocol 1) requires ~30 min for the preparation of the low-alkalinity copper reagent and the same amount of time for the preparation of the arsenomolybdate reagent. An additional 24 hr should be considered for the 37°C incubation when preparing the arsenomolybdate reagent. Therefore, a total of 26 hr is necessary from the beginning of the experiments to obtaining the results.

The Morgan-Elson assay (see Alternate Protocol 2) requires less than 1 hr for the preparation of all the reagents, but a one hour period is required for the reaction of the 2,4-pentadione solution with the amino-sugar samples. In conclusion, without considering the incubation time in the preparation of the reagents, 2 to 4 hrs working time should be enough to determine a sample concentration with any of the procedures.

In general, each method requires 1 to 2 hr for the manipulation involving the spectrometer and 1 to 2 additional hours for the manipulation involving the preparation of the reagents.

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The reference describes the analysis of reducing sugars using the Somogyi-Nelson method. It contains no experiments, but it is one of the first compilations explaining the method currently in use today.

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HPLC of Mono- and Disaccharides Using Refractive Index Detection

The choice of method for sugar analysis is dependent upon the composition of the sample and the mixture of sugars present. The main technique presented here is used for the determination of fructose, glucose, sucrose, maltose, and lactose in most foods, feeds, beverages, and nutritional products (see Basic Protocol). It is an efficient screening method for quality control and monitoring purposes and is used for fulfilling the nutrition labeling data requirements of some products. In addition, a protocol for isolating sugars (i.e., neutral compounds) from acids (e.g., citric acid) using anion-exchange mini-columns is described (see Alternate Protocol 1), along with a procedure for HPLC using calcium-loaded cation-exchange columns to overcome the difficulties in isolating sorbitol and galactose from glucose (see Alternate Protocol 2). Careful consideration of the matrix is required in order to determine which protocol is most appropriate. Because separation is accomplished using different mechanisms, the complete set of protocols allows analysis of a broad range of samples. The Basic Protocol exhibits a wide range of applicability, and the Alternate Protocols expand this range further. Data regarding the sugar composition of various foods can be found on the U.S. Department of Agriculture Web site, *http://www.nal.usda.gov/fnic/foodcomp*.

HPLC OF SUGARS USING AN AMINOPROPYLSILYL COLUMN

BASIC PROTOCOL

Sugars in the sample are extracted, dried, reconstituted in an aqueous acetonitrile solvent, and injected on a high-performance liquid chromatograph (HPLC) equipped with an aminopropylsilyl column and refractive index (RI) detector. Otherwise, standard laboratory equipment is used in conduct of this method. Development of this protocol was based, in part, on previous research conducted by Mason and Slover (1971) as well as methods developed by Brobst (1972) and Donnelly (1973). Work on carbohydrate analysis conducted by Brower (1966) was also considered. The aminopropylsilyl column is used due to its excellent sensitivity and ability to detect individual sugars at low concentration. Disadvantages include an incomplete separation of sorbitol and mannitol from glucose along with incomplete separation of galactose from glucose in some products (see Alternate Protocol 2). Maltitol interferes with maltose and lactose while inositol interferes with sucrose.

Due to interference, samples that are high in salt (especially sodium chloride) or samples with a high free amino acid content may be difficult to analyze using this method. In those cases, validated gas chromatography (GC) methods (Mason and Slover, 1971) are more applicable. In addition, samples containing sorbitol should be analyzed by GC or using a calcium-loaded anion-exchange column (see Alternate Protocol 2).

Materials

Sample or samples
0.28 M copper sulfate solution: store in a low actinic bottle up to 1 month at room temperature
1 N hydrochloric acid
1 N or 50% (w/v) sodium hydroxide
Acid-washed celite (J.T. Baker)
Nitrogen gas
Injection solvent: 50% (v/v) HPLC-grade acetonitrile in water
Reference standard solutions (see recipe)
Mobile phase (see recipe): 7:3 (v/v) acetonitrile/water

Contributed by Wayne Ellefson
Current Protocols in Food Analytical Chemistry (2002) E1.2.1-E1.2.9

200-ml volumetric flask
Whatman 2V filter paper
5-oz plastic cup and cap
4-dram vials with Teflon-lined screw caps
50°C heating block
2-ml injection vials
Syringe with 0.20-µm PTFE or nylon filter
High-performance liquid chromatograph (HPLC; Perkin-Elmer) with:
Isocratic LC pump 250
200 series refractive index (RI) detector
Advanced LC sample processor 1SS200
Analytical column: 250 × 4.6–mm aminopropylsilyl column (Supelco or equivalent)
Guard column: 10-cm aminopropylsilyl column (Supelco or equivalent; optional)

Prepare sample

1. Place 2 to 7 g of each sample in a separate 200-ml beaker. Add 40 ml deionized water and a magnetic stir bar to each. Place on a stirring plate and stir ~1 hr.

   A larger sample size is required for samples with low sugar content. A smaller sample size is required for samples with high sugar content in order to avoid solubility problems.

2. Add 10 ml of 0.28 M copper sulfate while stirring.

3. Adjust sample pH to 6.4 using 1 N hydrochloric acid or 1 N or 50% sodium hydroxide and a pH meter.

   Excess chloride can cause interference. Therefore, when titrating with hydrochloric acid, it is important to not overshoot the 6.4 pH.

4. Carefully transfer the sample to a 200-ml volumetric flask. Bring to volume with deionized water and mix well.

5. Filter the sample through Whatman 2V filter paper overlaid with ~0.5 g acid-washed celite (to aid filtration) into a 5-oz plastic cup with cap.

6. Pipet appropriate aliquots (8.0 and 1.0 ml) from the filtrate into separate 4-dram vials. Dry aliquots down under nitrogen gas for ~8 hr in a 50°C heating block.

   The 8-ml aliquot corresponds to the assay's lowest confidence level (<0.1%). The 1-ml aliquot is used to quantitate any of the sugars that are present in amounts greater than 1%. Adjustments in initial weights, volumes, or dilutions can be made in order to bring the sugar response on scale.

7. Add 5.0 ml injection solvent (50% acetonitrile) to each 4-dram vial and tighten the Teflon-lined screw cap. Vortex the sample vials every 10 to 15 min until no residue is found on the wall of the vials (~1 hr).

8. Filter into a 2-ml injection vial using a syringe and 0.2-µm PTFE or nylon filter.

   The clear solution is ready to be analyzed against reference standards using HPLC.

Prepare calibration standards

9. For each reference sugar, prepare a set of calibration standards in duplicate using the stock and working reference standard solutions.

   This gives five sets of calibration standards (i.e., fructose, glucose, sucrose, lactose, and maltose) each at concentrations of 0.05, 0.1, 0.3, 0.5, and 1.0 mg/ml.

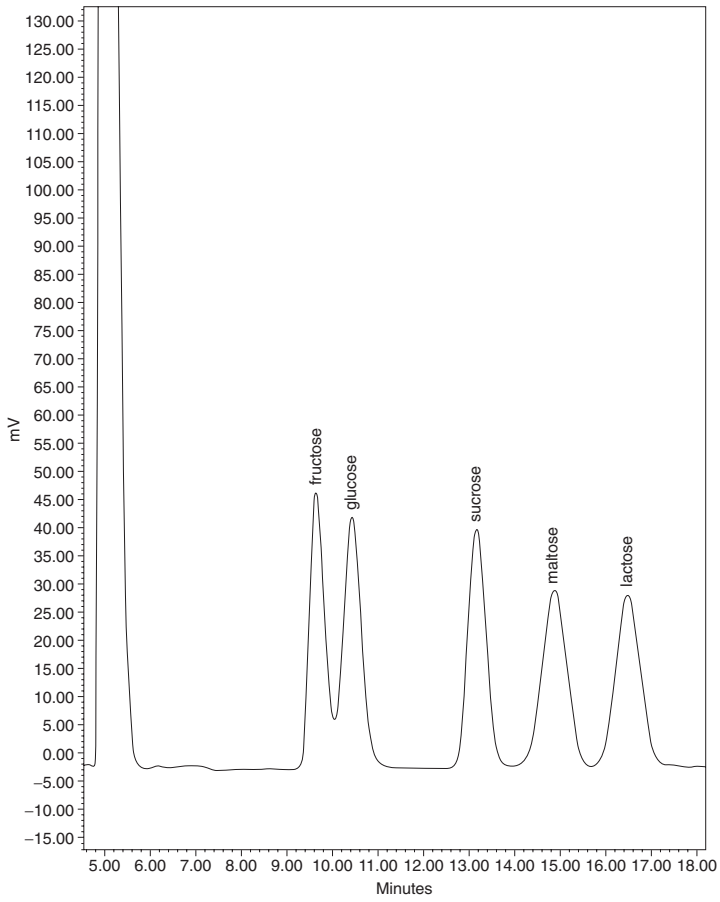

Figure E1.2.1 Chromatogram of a sugar standard analyzed as described (see Basic Protocol).

Perform chromatography

10. Analyze calibration standards and samples by HPLC with refractive index detection using the following conditions:

    Mobile phase: 7:3 (v/v) acetonitrile/water
    Flow rate: 1.0 ml/min
    Column temperature: ambient
    Elution mode: isocratic
    Run time: 25 min
    Injection volume: 75 µl.

    A run is typically composed to 55 to 60 injections, including replicate samples (aliquots in step 7), standards, and a minimum of 10% quality assurance samples, including duplicate analyses, validated control samples, or recoveries (see Critical Parameters).

    Newly prepared standards should be checked against previously qualified standards every six months. New standards are acceptable if the difference between standards is ±3.0% of the established values. Sample chromatograms are given for a sugar standard (Fig. E1.2.1), presweetened cereal (Fig. E1.2.2), ice cream (Fig. E1.2.3), and a meal replacement bar (Fig. E1.2.4).

    The aminopropylsilyl guard column is required for complex matrices with low sugar content. Simple matrices with high sugar content do not require the guard column. Other parameters may need adjustment in order to optimize the chromatography.

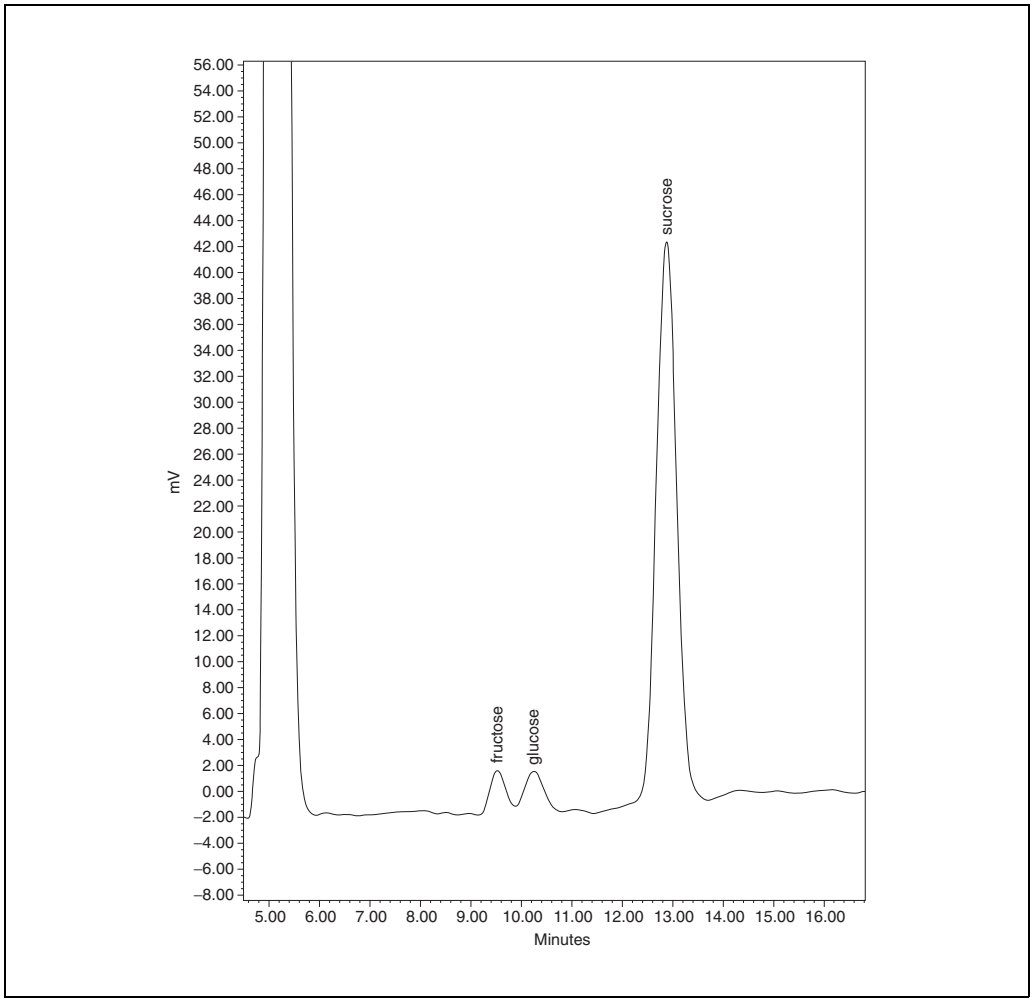

Figure E1.2.2 Chromatogram of a presweetened cereal analyzed as described (see Basic Protocol).

Analyze data

11. Generate five calibration curves (i.e., each sugar standard) by plotting a first-order curve of integrated peak area versus concentration (mg/ml) and performing linear regression analysis.

    The correlation coefficient (R value) should not be <0.999.

12. Determine the concentration (c in mg/ml) of each sugar in the sample by extrapolating from the appropriate calibration curve as a function of area response.

13. Determine the weight percent of each sugar in the sample using the equation:

$$\% \text{ sugar} = \frac{c \times V \times D \times 100}{w \times 1000 \text{ mg/g}}$$

    where V is the initial volume, D is the dilution factor (can vary according to the amount of each sugar that is present), and w is weight of the sample in grams (step 1).

14. Compare sample results against expectations and compare control sample results against the acceptable range (see Critical Parameters). Evaluate replicate results and recoveries for acceptability. Expectations are based upon historical data with a specific matrix, standard references, or expected results (e.g., claims). Acceptable ranges are determined during method validation.

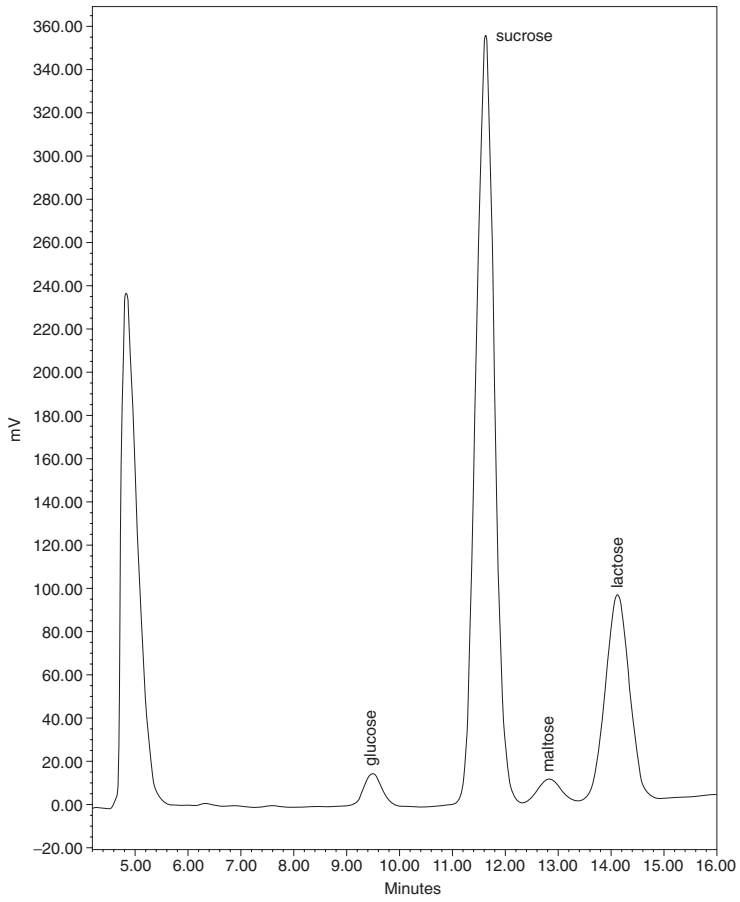

Figure E1.2.3 Chromatogram of an ice cream analyzed as described (see Basic Protocol).

If any of these do not match established acceptability criteria, the entire analytical run must be investigated. Acceptability criteria will change depending upon the sample matrix and the level of sugar anticipated. Based upon the investigation, another round of analysis may be necessary. If analysis is undertaken again, multiple replicates should be analyzed, taking into account the facts uncovered in the investigation.

SAMPLE PREPARATION USING ANION-EXCHANGE MINI-COLUMNS

ALTERNATE PROTOCOL 1

Sample matrices with high levels of citric or other organic acids (e.g., cranberry juice) can result in poor resolution. This method of sample preparation isolates sugars (i.e., neutral compounds) from acids using anion-exchange mini-columns. Development of this protocol is based on research conducted by Hong and Wrolstad (1986). Although the procedure does require additional time (i.e., twelve samples per hour), it does result in improved resolution and a more stable baseline.

Additional Materials *(also see Basic Protocol)*

Juice sample
7 mg/ml mannitol internal standard (Mallinckrodt)
Activated Sep-Pak C18 cartridge (Waters)
10-ml polyprep column containing 1.5 ml hydrated BioRex 5 anion-exchange resin (Bio-Rad)
0.45-µm Millipore filter (type HA)

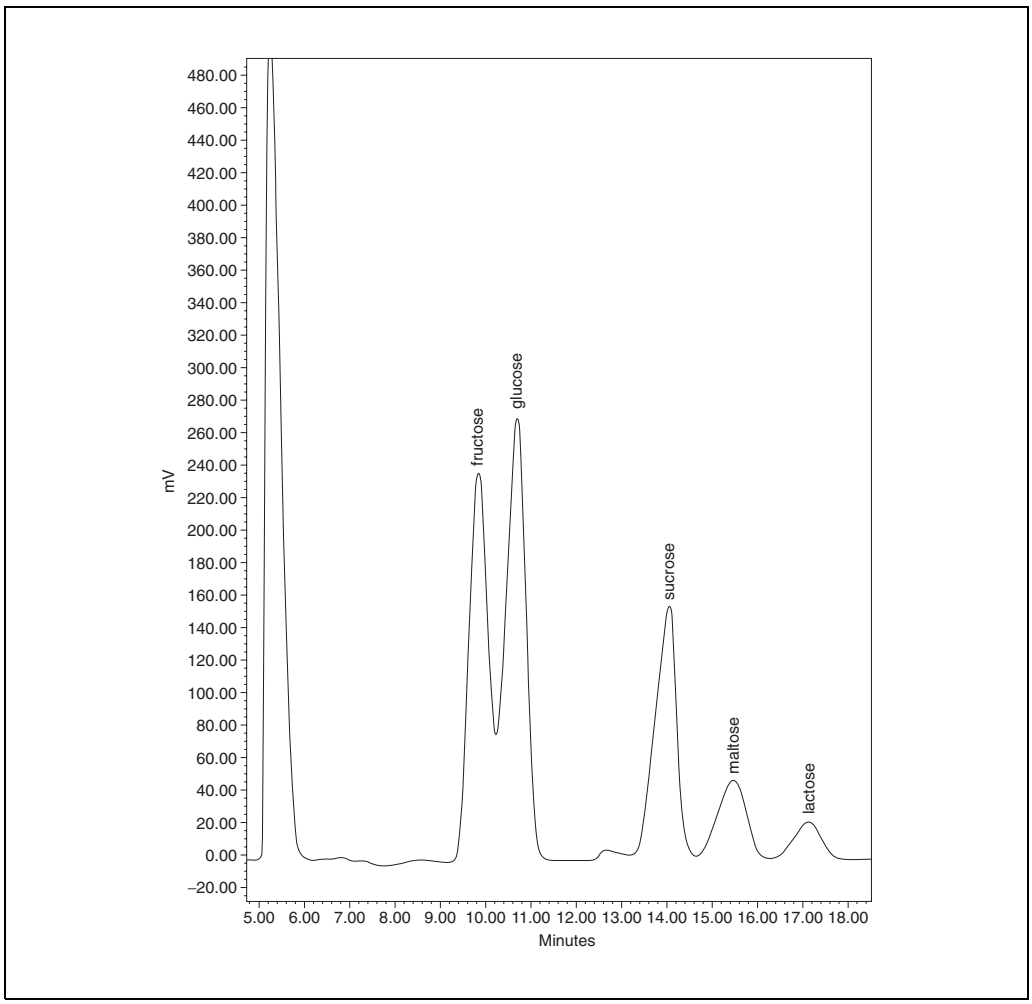

Figure E1.2.4 Chromatogram of a meal replacement bar analyzed as described (see Basic Protocol).

1. Mix 5 ml juice sample with 5 ml of 7 mg/ml mannitol internal standard solution and pass through an activated Sep-Pak C18 cartridge.
2. Discard the first 2 to 3 ml and apply the remaining eluate to a 10-ml polyprep column containing 1.5 ml hydrated BioRex 5 anion-exchange resin.
3. Discard the first 2 to 3 ml, collect remaining eluate, and filter through a 0.45-µm Millipore filter (type HA).
4. Inject 50 µl into the HPLC system. Include standards, prepare calibration curves, and analyze data (see Basic Protocol).

ALTERNATE PROTOCOL 2

HPLC OF MONO-AND OLIGOSACCHARIDES IN FRUIT JUICES USING A CALCIUM-LOADED CATION-EXCHANGE COLUMN

A disadvantage of the amino-bonded column (see Basic Protocol) is its limitation with samples containing small amounts of sorbitol in the presence of large quantities of glucose and the incomplete separation of both sorbitol and galactose from glucose. As an alternative, the use of an HPLC system with a calcium-loaded cation-exchange column can alleviate this constraint. Development of this protocol is based on research conducted by Durst et al. (1995). This application has been found to be useful for the determination of glucose, fructose, sorbitol, and sucrose in processed fruit products, and is commonly

used for analysis of fruit juices. The mechanism of separation is based on the strength of the bonding between *cis*-glycols of the sugar molecules with the Ca^{2+} loaded on the column. The elution order is related to the number and strength of *cis*-glycol complexes formed. The procedure is performed using a 7.8 × 300–mm Aminex HPX 87C column fitted with a 4.6 × 40–mm carbo-c Micro-Guard precolumn (both from Bio-Rad) and is identical to the Basic Protocol, except that the mobile phase is 0.2 mg/ml $Ca(NO_3)_2$, the flow rate is 0.7 ml/min, and the column temperature is 85°C.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Mobile phase

Using a graduated cylinder, measure 300 ml deionized water into a vacuum flask. Add 700 ml HPLC-grade acetonitrile to the flask. Mix solution with stir bar and magnetic stir plate. Degas the mobile phase daily in an ultrasonic bath under vacuum or using an in-line degasser. Prepare fresh for each analytical run.

Reference standard solutions

1.0 mg/ml stock solution:

Dry ACS-grade sugars (fructose, glucose, sucrose, lactose, and maltose; Sigma) 6 hr in a 60° ± 3°C vacuum oven. Weigh 0.2500 g of each sugar, adjusting the weight according to standard's purity (as supplied by the manufacturer) and the water present in the crystals. Transfer quantitatively into separate 250-ml volumetric flasks. Add 125 ml ultrapure water (e.g., Milli-Q-purified) to the flasks and completely dissolve the sugars. Bring the standard solutions up to volume with acetonitrile and mix thoroughly (final 50% acetonitrile). Store up to 6 months at room temperature.

0.05, 0.1, 0.3, and 0.5 mg/ml working solutions:

Pipet 5.0, 10.0, 30.0, and 50.0 ml of each 1.0 mg/ml stock solution into four different 100-ml volumetric flasks. Dilute to volume with injection solvent (50% acetonitrile) and mix well. Store up to 6 months at room temperature.

COMMENTARY

Background Information

The analytical methodology for the determination of sugars has improved dramatically and is continually evolving. The traditional wet-chemistry and enzymatic assays that detected classes of sugars (e.g., reducing sugars) have largely been replaced by chromatographic methods utilizing both gas-liquid (GLC) and high-performance liquid (HPLC) chromatography. These methods offer high specificity and the ability to detect several sugars at the same time, and have been actively developed over the past two decades. The HPLC technique is the official AOAC International method for routine sugar analysis (AOAC, 1995).

Refractive index (RI) detection is based on the principle that a light beam is refracted differently depending on the substance through which passes. A beam passing through a solution of water and sugar will refract more than a light beam passing through water alone. When an HPLC detector is set up so that a light beam passes through a cell with pure mobile phase and a flow cell with chromatographically separated analytes moving through it, the difference between the respective refractions can be measured and expressed as an electronic response. This detection method provides excellent sensitivity and the ability to achieve better precision at low levels of sugar content. UV detection is not commonly used for sugar analysis due to the greater potential for interference in the wavelength range required.

The most current advance in sugar analysis is the use of high-performance anion-exchange chromatography (HPAEC). This method, more commonly known as ion chromatography, is used for routine sugar profiles as well as a

variety of unique sugar or sugar alcohol analytical situations. Ion chromatography tends to use different mobile phases than HPLC with a calcium-loaded column. In addition, a different detection system (pulse amperometric detection) is commonly used with ion chromatography. This provides another tool for use with difficult samples.

Critical Parameters

Control samples are used as a quality control tool. These samples have a certified or validated level of the analyte of interest. Inserting a control sample into each analytical run provides a tool by which to judge the overall acceptability of the run. Appropriate control samples must be developed prior to conducting any sample analysis. National Institute for Standards and Technology (NIST) Standard Reference Materials (SRMs) may be used in the study or to assist in the validation of in-house control samples. In-house control samples may include items such as fortified breakfast cereal, beverage products, milk powder, candy, or other shelf-stable foods that contain sugar. Preparation of the control sample includes grinding and subsequent analysis of the sugars present. It is prudent to develop both high- and low-level control samples encompassing all the sugars included in the profile. The control range should be established with a minimum of sixteen replicate analyses conducted over multiple days. Spike recoveries should be conducted at 0.5×, 1×, and either 1.5× or 2× of the mean (depending upon the level of sugar). The data are often verified by the use of another method (e.g., Alternate Protocols). Statistics are applied to determine the acceptable range for results.

The solubility and homogeneity of the sample are key to accurate analysis. This solubility can be affected by other ingredients in the product to be analyzed. Certain situations may require use of a much smaller sample size to account for solubility issues. Proper steps must be taken to grind or homogenize the sample to a requisite uniformity. The various HPLC columns typically last for at least 500 injections, sometimes as many as 1000 injections. Schiff's base formation with sugars does slightly limit the life of the aminopropylsilyl column. Many column manufacturers list column regeneration procedures. These procedures may extend the column life somewhat.

Troubleshooting

The HPLC sugar assay was developed and validated as an efficient screening technique. As a result, some limitations may be encountered. Samples high in salt content may create difficulties, particularly interfering with glucose or sucrose. In some cases, some of the salt can be removed with an HPLC clean-up column or the use of a column different from the one specified (e.g., anion-exchange or calcium-loaded column). The preferred solution is to analyze such samples using GC instead of HPLC.

Fructose and glucose elute close to one another, which, depending upon the amounts present, may cause chromatographic overlap in some matrices. HPLC chromatographic peaks for the five common sugars are not always as sharp as is desirable. This can lead to different results amongst scientists, depending upon how the baseline is drawn. In some cases, an adjustment of the chromatographic parameters (e.g., mobile phase) is necessary.

High levels of citric acid may cause problems with fructose or glucose. In these cases, the use of anion-exchange minicolumns (Alternate Protocol 1) or GC can be used to overcome this problem. For samples that are high in lipid content (e.g., dairy products) and are thus difficult to dissolve, use warm water for dispersion.

Anticipated Results

Typical retention times for the common sugars are shown in Table E1.2.1. These times may vary in laboratory applications. Reproducibil-

Table E1.2.1 Typical Retention Times of Common Sugars on Supelco Columns

Sugar	Retention time (min)	
	Amino column	Calcium column
Fructose	8.3	14.9
Glucose	9.8	12.0
Sucrose	14.0	9.8
Maltose	17.4	9.8
Lactose	19.5	10.2

ity among duplicates should be high on products with significant sugar levels. In matrices containing very low levels of sugar, the relative reproducibility (%RSD) will not be as good. This method is intended for routine sugar analysis. It is not intended for products containing less common sugars, or for sugar alcohol analysis. These products are better analyzed using gas chromatography.

Time Considerations

A typical run of ~20 samples requires 2 days to complete the analysis process. Sample preparation (i.e., weighing, mixing, adjusting pH, adjusting volume, and aliquotting) takes ~4 hr. Two or three aliquots are taken from each sample and each aliquot is injected once. The number of aliquots needed is dependent upon the percentage of individual sugars (i.e., 0.1% to 1.8%, 1.0% to 20%, and >20%) contained in the sample. For example, the higher the differential between the individual sugars, the more aliquots are required. Aliquots should be allowed to dry for ~8 hr. After drying, at least 1 hr is needed to reconstitute them in injection solvent. The run is composed of 55 to 60 injections including the standards, and each injection takes 17 to 25 min. The total run time is ~20 hr.

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Mason, B.S. and Slover, H.T. 1971. A gas chromatographic method for the determination of sugars in foods. *J. Agric. Food Chem.* 19:551-554.

Key References

AOAC, 1995. See above.

The AOAC methods are considered the official assays to be used for nutrition labeling in the United States. The methods are adopted only after a rigorous collaborative study.

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Overview of Laboratory Isolation of Starch from Plant Materials

Starch, a glucose biopolymer, is the major storage component of most economically important crops: cereal (e.g., wheat, rice, corn, oat, barley), legume (e.g., lentil, bean, pea), tuber (e.g., potato, sweet potato), and yam (*Dioscorea*). It exists as water-insoluble granules, generally ranging in size between 3 and 60 μm. Starch has many uses in both food (e.g., binder, bulking agent, texture modifier, fat replacer) and nonfood (e.g., paper, cardboard, mining, pharmaceutical) industries. Physicochemical properties of starch differ with its source. The characterization of these properties and their functionality has been an important area of research in starch chemistry and technology. The data from such research have been critical in evaluating the quality of a starch in order to define its suitability for a specific industrial application and economic value. Successful characterization of starch depends greatly upon the purity of the isolate. A good representative sample should contain >96% (w/w) starch (*UNIT E2.2*) and be devoid of other plant components, such as fiber (soluble and insoluble), protein, and lipids. These impurities, especially soluble gums, proteins, and lipids, influence starch properties and lead to false characterization.

Because plant materials differ in their tissue structure and composition, many different protocols for starch isolation have been established. Therefore, it is difficult to recommend a universal method. However, all established protocols were based on either of the following two procedures for starch isolation: (1) grain steeping/wet grinding/starch recovery (Watson et al., 1955; Banks and Greenwood, 1975) or (2) dough making/dough washing/starch recovery (Wolf, 1964). This unit presents a general outline for starch isolation with emphasis on special considerations that are required to handle different plant materials. Because starch granules are insoluble in water and have a high density (~1.5 g/ml), most protocols for laboratory starch isolation from plant materials have used excess water to slurry or wash starch, and centrifugation to separate starch from other plant components. Figure E2.1.1 outlines the basic steps of starch isolation. Discussed below are some specific problems encountered at each basic step involved in the isolation of starch from different plant materials. Also included are recommended measures to avoid or minimize problems during isolation, ensuring an increased reliability of both quantitative and qualitative recovery of starch from a particular source.

RAW MATERIAL PREPARATION

The raw material should be clean and devoid of contaminants such as stones, soil, bad seeds, tubers, roots, yams, and foreign plant residues (e.g., stem, stalk, leaf). Metal screens and air aspiration systems can be used to remove most of these contaminants. Soil particles adhering to plant material may be removed by scouring and water washing. However, washed material should be dried (if it is to be dry ground into flour) to ≤10% moisture content (*UNIT A1.1*), as higher moisture levels may lead to problems during dry grinding and storage. Drying should be done gradually at low temperatures because high temperatures (>40°C) have been shown to alter the physicochemical properties of native starch (Hoover and Vasanthan, 1994a,b).

Hulled grains (cereals and legumes) are usually de-hulled, whereas tuber, root, and yam tissues are hand peeled (without damaging or losing the pulp) and diced prior to starch extraction. Hard plant materials (i.e., grains) are usually ground into flour by simple laboratory dry-milling techniques (Fig. E2.1.1, route D). However, it is important to know that the dry milling of hard plant materials can result in substantial starch granule damage (as determined by an enzymatic procedure approved by the American Association of Cereal Chemists), especially in wheat grains (up to 12% reported) where starch granules are well integrated with gluten proteins. For most of the other grains (i.e., barley, oat, rice, corn, legumes), starch granule damage of up to 5% has been reported. A high percentage of damaged starch granules may alter the physicochemical properties of starch.

Steeping (Fig. E2.1.1, route B) softens hard plant materials, especially grains, and facilitates wet grinding, thus minimizing starch granule damage. Steeping times range from 4 to 16 hr depending on the hardness of the plant material. Steeping or dry grinding are unnecessary steps in starch isolation from tuber, root, and yam because they are softer plant materials with high moisture contents, and thus can be

Contributed by Thava Vasanthan

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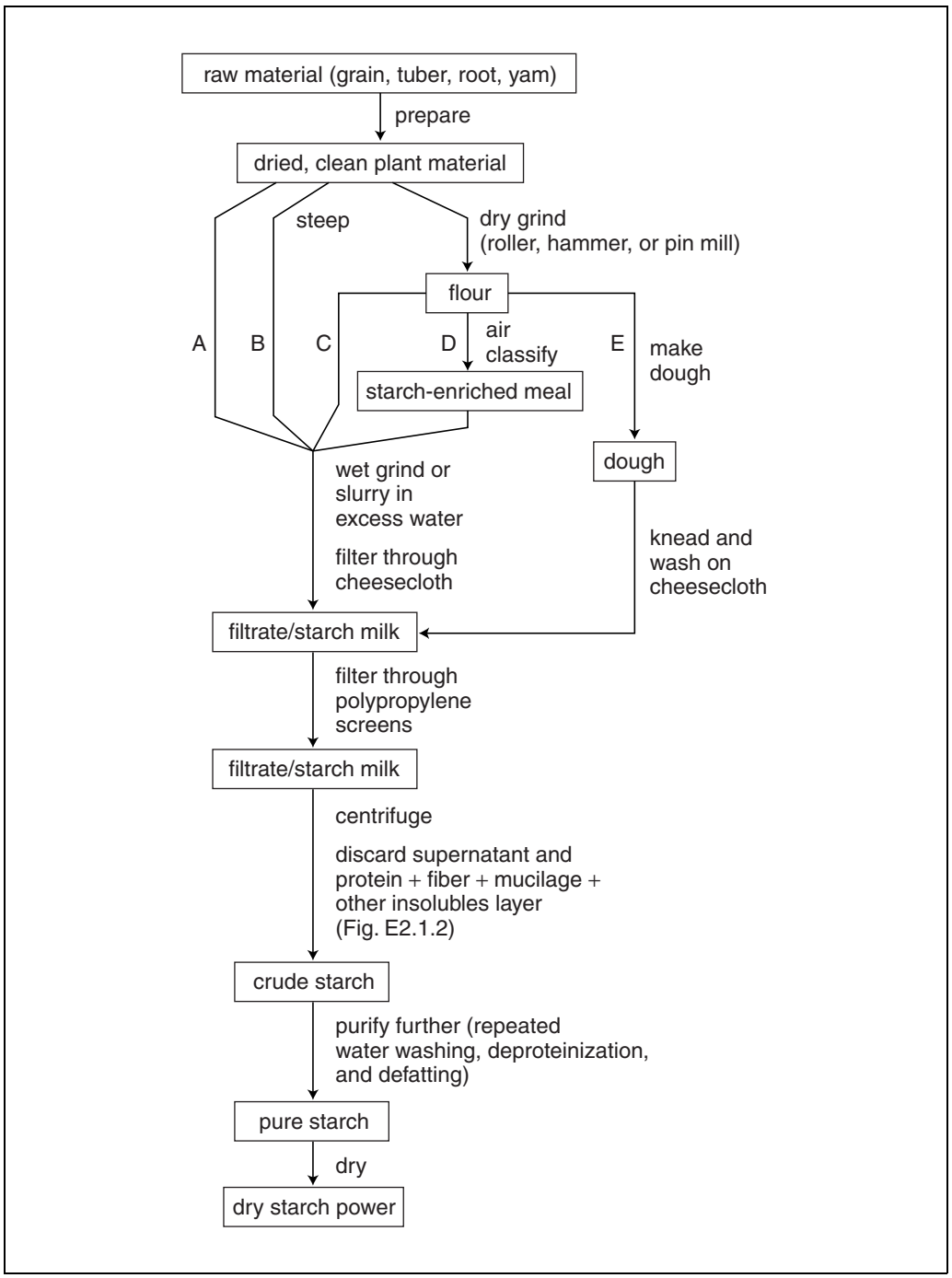

Figure E2.1.1 Basic steps in starch isolation from plant materials. Route A is for tubers, roots, and yams; routes B-D are for grains; route E is for wheat. See text for more details on individual steps.

peeled, sliced into small pieces, and directly slurried in water using a blender (Fig. E2.1.1, route A). Morrison et al. (1984) reported that good recovery of wheat starch was ensured by lightly cracking the kernels, steeping in acid, neutralizing, and treating with a protease prior to slurrying and further processing.

WET GRINDING OR SLURRYING OF PLANT MATERIAL

As illustrated in Figure E2.1.1, different approaches (routes A to E) for slurrying the plant material in water have been reported in the literature. Route A involves direct slurrying of plant material in water, which is usually practiced for soft plant tissues (i.e., tuber, root, and yam). Routes B, C, and D are usually practiced for hard plant tissues (i.e., cereal and

legume grains). Route E, which is known as "dough washing" or the Martin process (Knight, 1984), is specifically used for isolation of starch from wheat flour (produced by roller or pin-milling technique).

For water slurrying (Fig. E2.1.1, routes A to D), the ratio of plant material to water is often 1:>10 (w/w; water requirement for slurrying of different plant materials is discussed below). The weight of plant or raw material that should be taken for starch extraction will depend on the starch requirement, starch content of the plant material, and starch recovery of the extraction procedure (usually 85% to 90%). For example, 100 g of fresh peeled potato tuber, corn grains, and barley grains usually yields 15 to 16, 55 to 60, and 40 to 45 g of starch, respectively. The blending of plant material and water should be carried out until a smooth slurry forms (usually 5 to 10 min). For grain flours (e.g., oat, barley, corn) water slurrying can be done in a beaker using an overhead stirrer at high speed. The blending process generates heat, and therefore care must be taken (crushed ice can be added into the slurry) to avoid heat-induced damage to starch granules. Adding 0.01% (w/v) sodium metabisulfite or 0.01 M (final concentration) mercuric chloride is highly recommended during slurrying. Either of these compounds will inhibit microbial growth and the activity of amylose (a commonly occurring plant enzyme that hydrolyzes starch).

The water requirement for slurrying differs with the source of plant material. Cereal grains contain water-soluble nonstarch polysaccharides such as β-glucan and hemicellulose, which are referred to as gums. These gums show high water-binding capacity, which substantially increases the viscosity of the slurry, making the filtration process difficult. They also slow down the sedimentation of plant components during centrifugation (Fig. E2.1.1). A large amount of water (1:>25, w/w) is necessary for starch isolation from plant materials containing these gums. Some barley varieties contain large amounts (up to 10%, w/w) of these nonstarch polysaccharides, with decreasing levels in oat, wheat, rice, and corn, respectively. However, several procedures have been developed to reduce slurry viscosity. The use of enzymes such as cellulase, β-glucanase, and xylanase to hydrolyze nonstarch polysaccharides has been investigated (Zheng and Bhatty, 1998).

Vasanthan and Bhatty (1995) reported the use of pin milling and air classification as a technique for preconcentrating starch prior to wet isolation (Fig. E2.1.1, route D). Air classification is a technique generally used to separate heterogeneous particles (<500 μm in size) in a dry-powder system into subgroups of fairly uniform particle size. The subgroups or powder fractions are generally referred to as "fine" and "coarse" in relation to the set particle size. Studies on air classification of grain flours from cereal (wheat, barley, barley-malt, corn, oat, rye, triticale, sorghum, and rice) and legume or pulse (e.g., field pea, lupine, chickpeas, navy beans) were reported. The grinding of seed grains into flour by hammer or pin or attrition (i.e., roller) milling, followed by air classification, separates and concentrates the seed constituents (e.g., starch, protein, fiber) into coarse and fine flour fractions. Air classification separates flour particles on the basis of differences in density, mass, and particle size or projected area in the direction of air flow. Depending on the granule size, starch can concentrate into one of these fractions. Small (<10 μm in diameter) and large (>10 μm in diameter) granules usually concentrate with fine and coarse fractions, respectively. However, an air classifier is not commonly available in laboratories due to its high cost. Laboratory-scale air classifiers can be purchased from Hosokawa Micron Powder Systems.

Legume seeds contain insoluble flocculent proteins, which decrease sedimentation of starch and also cosettle with starch to give a brownish deposit (Schoch and Maywald, 1968). This material requires further starch purification (as discussed below). Tubers and roots contain relatively low amounts of nonstarch polysaccharides or flocculent proteins, and therefore require less water during starch extraction. A direct slurrying of steeped grain or dry flour in aqueous alkali (0.01 to 0.05 N NaOH or KOH) can also be carried out to enhance the solubility of gums and proteins. However, neutralization of the residual alkali in the starch isolate using a mild acid solution (0.1 N HCl) is required at the end of the isolation process.

Dough washing (Fig. E2.1.1, route E), or the Martin process, is a popular method for wheat starch isolation. The process involves dry grinding the wheat to produce flour, adding water to make dough (flour/water ratio is 2:1, w/w), and washing the dough with excess water while kneading on double-layered cheesecloth in order to wash out starch granules from the gluten protein network. The washing should be carried out until the milkiness (whiteness) of the filtrate disappears or is at a minimum. The starch in the filtrate (starch milk) will then be

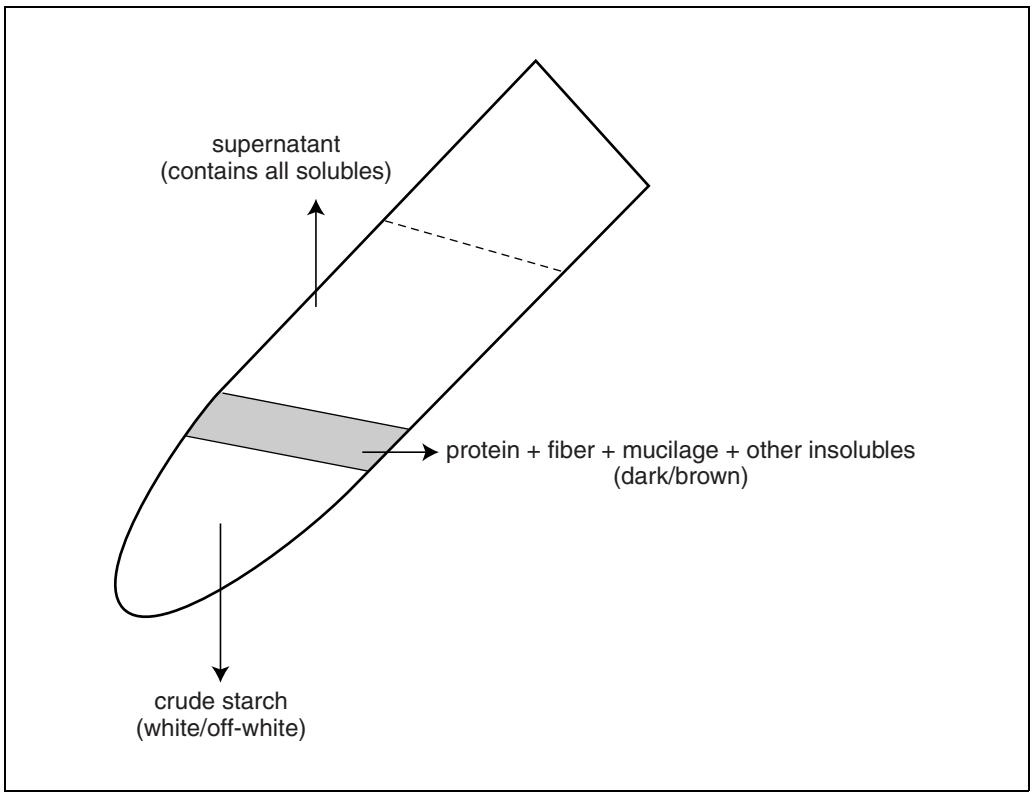

Figure E2.1.2 Sedimentation of plant components during centrifugation.

further refined and recovered using the process outlined in Figure E2.1.1: filtration through a series of polypropylene screens (250, 175, 125, and/or 75 μm), centrifugation, and so on. Wheat starch processed using this technique can show up to 12% starch damage.

FILTRATION

In the slurrying processes (Fig. E2.1.1, routes A to D), the first filtration is usually done using double-layered cheesecloth in order to screen out large water-insoluble fiber particles. During the filtration step, starch is washed away by spraying water (from a wash bottle) onto the residue. The lack of milkiness of the filtrate indicates adequate washing. Vigorous squeezing of the cheesecloth in order to accelerate the filtration process should be avoided. Carrying out at least two more rounds of grinding and filtration using the residue left on the cheesecloth will improve the recovery of starch. The resulting filtrate containing medium and small fibers is then subjected to further filtration. A series of polypropylene screens (250, 175, 125, and/or 75 μm) is normally used. The filtration process yields a milky filtrate that is rich in starch but contains other plant components, such as gums or mucilage, protein, and fine fiber particles, that can be separated out by centrifugation.

CENTRIFUGATION

Centrifugation is an important step in starch isolation that separates starch from fine fibers, insoluble or soluble protein, and gum or mucilage compounds. Centrifugation (usually 20 to 30 min at 5000 to 8000 × *g*, 20°C) settles the starch (a white layer) at the bottom of the centrifuge tube (Fig. E2.1.2). The water-insoluble contaminants (e.g., protein, fine fiber) form a dark or brown layer on top of the starch layer. This contaminant layer is normally removed manually by scraping with a spatula, being careful not to scrape away any of the starch layer.

Starch granules usually exist in a range of sizes. Small-granule starches (also referred to as B-starch, with an average diameter between 4 and 5 μm) from plant materials (i.e., barley, wheat, rice, oat, quinoa, buckwheat, and canary seed) are difficult to recover because they usually co-sediment with the protein, fiber, and mucilage layer (dark or brown layer) during centrifugation. In order to enhance starch recovery, small-granule starch from this layer must be recovered by protease treatment followed by toluene shaking (McDonald and Stark, 1988). For protease treatment, 5 g (wet weight) dark or brown layer is mixed with 150 ml of 0.1 M Tris·Cl (pH 7.6) containing 0.5% (w/v) $NaHSO_4$, 0.01% (w/v) thimerosal, and

25 mg proteinase K, followed by incubation at 25°C for 24 hr. The digest is then centrifuged (10 min at 5000 × *g*, 20°C) and the supernatant discarded. The pellet at the bottom of the centrifuge bottle is suspended in 500 ml of 0.2 M NaCl containing 14% (v/v) toluene for 12 hr at 20°C and then centrifuged (10 min at 5000 × *g*, 20°C). The supernatant is discarded, and the starch pellet at the bottom of the centrifuge tube is dried and added back to the original starch mass. A combination of cellulase and protease treatments would further enhance the recovery of small starch granules.

Tuber, root, and yam starches usually show a bi- or trimodal granule size distribution. Quantitative recovery of these starches is usually not a difficult task because the small starch granules in these plant sources show an average size of 11 to 12 μm, which is large enough to separate from other grain components during centrifugation. The legume starches are usually large and highly uniform in size, making their quantitative recovery a less difficult task.

Vasanthan and Bhatty (1995) reported a protocol for isolation of prime barley starch (a large-granule starch sometimes referred to as A-starch). In this protocol, barley was pin milled and air classified to obtain a starch-enriched meal fraction that contained mainly prime starch. Further purification of this meal fraction by slurrying in excess water, screening through a 40-μm polypropylene mesh to remove fibrous material, and washing with an excess amount of aqueous 0.05 N NaOH to remove protein yielded prime starch. A pilot-scale investigation of this process (Vasanthan et al., 1997) suggested that bran removal by pearling of grains (which removes ~15% to 17% of grain weight) before pin milling and air classification would substantially reduce fine-fiber contamination in the isolated starch mass.

FURTHER PURIFICATION OF CRUDE STARCH

Morrison (1981, 1988) reported that nitrogen content of isolated starches represents contamination from storage proteins; lipids that contain choline, ethanolamine, and serine; and proteins located on the surface and inside the starch granules. Tubers, roots, and yams contain very little protein (<3.0%, w/w, dry weight) and lipid (<1%, w/w, dry weight) compared to legume and cereal grains, which minimizes the contamination of starch by these components during isolation. Washing once with toluene or aqueous alkali (0.05 N NaOH or KOH) is adequate. However, repeated washing with aqueous alkali is highly recommended for cereal and legume starches. Washing can be done by vigorously shaking starch and alkali (1:10, w/v) in a centrifuge bottle, centrifuging (10 min at 5000 × *g*, 20°C), and discarding the supernatant. Lipids associated with the isolated starch have been found to occur on both the surface and the inside of granules (Morrison, 1981). Surface lipids are usually nonstarch lipids that are absorbed into the surface layer of starch granules during isolation. Excess contamination by nonstarch lipids would alter the physicochemical properties of starch granules and should be removed by washing with a solvent blend such as chloroform/methanol (2:1, v/v; Morrison, 1981; Vasanthan and Hoover, 1992). The recommended starch/solvent ratio is 1:20 (w/v), and the extraction should be carried out at temperatures <30°C.

DRYING OF PURE STARCH

Pure starch cake, recovered by centrifugation (Fig. E2.1.1), contains >30% moisture. This starch is often dried by one of the following three methods: oven drying at temperatures between 30° and 40°C, freeze drying, or washing on a glass filter with excess ethanol. During oven drying, the temperature should not exceed 40°C because starch undergoes a process called annealing when subjected to high temperatures (>50°C) and moisture (>10%, w/w), which alters its properties (Whistler et al., 1959; Hoover and Vasanthan, 1994a,b). Spray drying is another potential technique, but is seldom used due to the exposure of starches to temperatures >50°C during drying.

The dried starch should be hand ground to a powder using a mortar and pestle, sieved (250 μm), and stored in a tightly closed container under dry conditions. The color of the pure starch is bright white. The purity of the isolated starch must be determined (*UNIT E2.2*) and noted on the label with other information such as pH, moisture, plant source, variety, date of extraction, and the name of the person who performed the extraction.

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Enzymatic Quantitation of Total Starch in Plant Products

Starch is one of the storage materials in higher plants. Quantitatively, it is the most important component of economically important grains (cereals and pulse or starchy legumes), tubers (potato), roots (tapioca), and yams (e.g., *Diascorea* species) and their food products, such as flour, whole-grain meal, bran, bread, pasta, noodles, potato chips, and french fries. A method of total starch determination with high accuracy, precision, and rapidity is important in both a research (e.g., proximate composition, purity check) as well as a commercial (e.g., nutrition labeling, purity check, pricing, and selection, or screening of plant products used in starch production) context.

This unit describes two methods for determining total starch: a Basic Protocol originally established by Megazyme International Ireland (McCleary et al., 1997a,b) and an Alternate Protocol established by Holm et al. (1986). Both protocols are based on the selective conversion of starch in a sample to glucose by a combination of amylase enzymes, followed by measurement of glucose using a colorimetric technique. Overestimation is unlikely as these amylase enzymes are starch specific; however, the quantitative conversion of starch to glucose must be ensured in order to avoid underestimation.

The Megazyme procedure requires the company's Total Starch Assay Kit, which contains most of the necessary enzymes and chemicals. This procedure was approved by the American Association of Cereal Chemists (AACC Method 76-13; AACC, 1976) and the Association of Official Analytical Chemists (AOAC Method 996.11) following an extensive collaborative AACC/AOAC interlaboratory evaluation (McCleary et al., 1997b). The procedure is simpler than a previously reported method (AACC, Method 76-12). The procedure by Holm et al. (1986) described in the Alternate Protocol uses enzymes and chemicals purchased individually.

MEGAZYME TOTAL STARCH ASSAY (AMYLOGLUCOSIDASE/α-AMYLASE METHOD)

BASIC PROTOCOL

This protocol is based on the Megazyme Total Starch Assay Kit, which is suitable for measurement of total starch in most native and processed cereal products, as well as other plant materials. Hydrolytic conversion of starch to glucose is performed in two phases. In the first phase, starch, water, and thermostable α-amylase are mixed and heated to boiling. The starch is totally solubilized and becomes partially hydrolyzed to dextrins. In the second phase, the resulting starch dextrins are quantitatively hydrolyzed to glucose by amyloglucosidase. Samples containing high levels of resistant starch (e.g., high-amylose corn starch, pasta) require pretreatment with dimethyl sulfoxide (DMSO) at 100°C (see Commentary).

The kit includes necessary enzymes (thermostable α-amylase and amyloglucosidase), some reagents (buffer concentrate and glucose oxidase/peroxidase [GOPOD] reagent) and standards (glucose solution and corn starch) to carry out 100 starch determinations. The other reagents required for this procedure may be obtained from any chemical supplier.

Contributed by Thava Vasanthan

Current Protocols in Food Analytical Chemistry (2001) E2.2.1-E2.2.9

Materials

Sample
Total Starch Assay Kit (Megazyme International Ireland), containing:
  Starch Standard
  100 U/ml thermostable α-amylase (see recipe for dilution)
  200 U/ml amyloglucosidase (store at 4°C)
  1 mg/ml glucose standard
  Glucose oxidase/peroxidase (GOPOD) reagent (see recipe for dilution)
80% (v/v) ethanol
Dimethyl sulfoxide (DMSO; e.g., Sigma)
50 mM MOPS buffer (see recipe)
200 mM sodium acetate buffer (see recipe)

Grinding mill with 0.5-mm screen
16 × 120–mm round-bottom glass test tubes
50°, 80° to 85°C (optional), and boiling water baths
Positive-displacement pipettor (to dispense 0.1 ml)
100-ml volumetric flask
Spectrophotometer set at 510 nm

CAUTION: DMSO is a skin irritant; handle with care.

NOTE: Dilute thermostable α-amylase and prepare GOPOD reagent according to Total Starch Assay Kit instructions. Refer to kit instructions for storage conditions.

Prepare sample

1. Use a grinding mill with a 0.5-mm screen to prepare sample.

   UNIT E2.1 presents a detailed discussion of sample preparation for starch analysis.

2. Add ~100 mg sample (noting weight) to the bottom of a 16 × 120–mm glass test tube. Prepare a separate standard tube containing a starch standard. Process in parallel with sample.

   Samples should be analyzed in triplicate. A standard should be included with each set of determinations.

   Because the presence of preexisting glucose and maltodextrins in a sample can lead to overestimation, sample blanks can be run in parallel without treatment with amylase enzymes (step 3a) as a control. Alternatively, analysis of the sample blank can be avoided by preextracting samples with 80% ethanol (step 3b).

For comparison with a sample blank:

3a. Set up a test tube with an equal amount of sample and process in parallel with sample tube except where noted otherwise.

4a. To aid dispersion, add 0.2 ml of 80% ethanol to sample and blank, and vortex. Proceed to step 6 for hydrolysis of resistant starch, or else to step 7.

For removal of preexisting glucose:

3b. Mix sample with ~10 ml of 80% ethanol and heat 5 min in an 80° to 85°C water bath.

4b. Centrifuge 10 min at 1000 × *g*, 25°C, and decant ethanol without losing sample.

5b. Repeat steps 3b and 4b. Proceed to step 6 for hydrolysis of resistant starch, or else to step 7.

Hydrolyze starch to glucose

6. *Optional:* Solubilize resistant starch by adding 2 ml DMSO and heating 5 min in a boiling water bath.

   Resistant starch escapes hydrolysis by starch-specific enzymes (see Commentary). Studies have shown that resistant starch, upon treatment with DMSO, becomes solubilized and, thus, hydrolyzable by amylase enzymes.

7. Add 3 ml thermostable α-amylase (300 U) diluted in 50 mM MOPS buffer and vortex vigorously. Add 3 ml of 50 mM MOPS buffer instead of enzyme to sample blank.
8. Incubate 6 min in boiling water bath, stirring tube vigorously after 2 min and 4 min in order to ensure complete homogeneity of the slurry.
9. Place tube in a 50°C water bath. Add 4 ml of 200 mM sodium acetate buffer. Add 0.1 ml amyloglucosidase (20 U) using a positive-displacement pipettor. Add 0.1 ml water instead of enzyme to sample blank.

   Amyloglucosidase is viscous and should be dispensed with a positive-displacement pipettor.

10. Vortex tube and incubate 30 min at 50°C.
11. Transfer entire contents of the tube to a 100-ml volumetric flask, rinsing tube thoroughly with water. Bring to volume with water and mix thoroughly.

    If the anticipated starch content is low (i.e., 1% to 10% as in high-fiber material), use a 10-ml volumetric flask and adjust volume to 10 ml.

12. Centrifuge a 2- to 3-ml aliquot 10 min at $1000 \times g$, 25°C.
13. Transfer duplicate 0.1-ml aliquots of supernatant to the bottom of two 16 × 120–mm glass test tubes.

Prepare controls

14. Prepare glucose controls in quadruplicate by placing 0.1 ml of 1 mg/ml glucose standard (100 µg glucose) in each of four tubes. Process in parallel with sample tube.
15. Prepare a reagent blank by adding 0.1 ml water to a tube and processing in parallel with sample tube.

    The glucose standards and reagent blanks are prepared only for the GOPOD reaction and must be run with each set of samples.

Quantitate glucose

16. Add 3 ml GOPOD reagent to each tube (including glucose controls and reagent blank), mix, and incubate 20 min in the 50°C water bath.

    The time of incubation with GOPOD reagent is not critical, but should be between 20 and 60 min, and should be the same for all samples.

17. Transfer 1 ml from each tube into a disposable cuvette.
18. Zero a spectrophotometer set at 510 nm using the reagent blank, and then read the absorbance for each cuvette.
19. Calculate total starch in the sample as follows:

    $$\% \text{ Starch} = \Delta E \times F \times \text{DF} \times 1 \text{ mg}/1000 \text{ µg} \times 100/W \times 162/180$$

    where ΔE is the absorbance of the sample minus the absorbance of the sample blank, F is a factor for converting from absorbance to µg, DF is the dilution factor (from step 11), $100/W$ is a factor used to express starch as a percentage of flour weight (W) in mg, and the fraction 162/180 adjusts from free glucose to anhydro glucose, as occurs in starch. To calculate F, divide 100 (for the µg glucose) by the average absorbance of the quadruplicate glucose controls.

When the DF is 1000 (i.e., the final volume in step 11 was 100 ml), this can be more simply represented by the equation % Starch = $\Delta E \times F/W \times 90$.

20. Calculate total starch on a dry weight basis using the following equation:

    % Starch (dry weight) = % Starch × 100/(100 − %moisture content)

    For the determination of moisture content, see Chapter A1.

ALTERNATE PROTOCOL

ENZYMATIC QUANTITATION OF TOTAL STARCH (HOLM METHOD)

The protocol developed by Holm et al. (1986) was evaluated by analysis of starch content in wheat starch, white wheat flour, whole-grain wheat, and industrially processed wheat products. The major advantage of this protocol over the Basic Protocol is its reduced cost. The chemicals for the reagent preparations and the enzymes are purchased directly from the companies that produce them. The original method did not include RS3 (resistant starch) in its quantitation. A DMSO treatment step has been added (step 4) to solve this problem.

Additional Materials *(also see Basic Protocol)*

Standard flour or starch sample with known starch content
Thermostable α-amylase (120 U/g, 1.20 g/ml; Termamyl 120 L, Novo Nordisk Biochem or equivalent)
Amyloglucosidase from *Aspergillus niger* (140 U/ml in 3.2 M ammonium sulfate solution, Boehringer Mannheim or equivalent)
0.1 M sodium acetate buffer (see recipe)
100 µg/ml glucose standard solution
Glucose oxidase/peroxidase (GOPOD) reagent (see recipe)

40° and 60°C water baths
25-ml volumetric flasks
10 × 100–mm glass test tubes

Prepare starch sample

1. Mill sample to pass through a <0.5-mm screen.

   UNIT E2.1 presents a detailed discussion of sample preparation for starch analysis.

2. Add 250 mg sample to the bottom of a 16 × 120–mm glass test tube. For very high-starch samples, i.e., corn starch, use 20 mg. Prepare a separate standard tube containing a standard flour or starch sample whose starch content is known. Process in parallel. Run all samples in triplicate.

   A standard should be included with each set of determinations.

3. Wash with ethanol (see Basic Protocol, steps 3b to 5b).

   Ethanol washing is very important in order to remove any preexisting glucose and maltodextrins, which would lead to overestimation. Alternatively, a sample blank can be used (see Basic Protocol, steps 3a and 4a).

4. *Optional:* Mix with 2 ml DMSO and heat 5 min in a boiling water bath.

   Resistant starch escapes hydrolysis by starch-specific enzymes (see Commentary). Studies have shown that resistant starch, upon treatment with DMSO, becomes solubilized and, thus, hydrolyzable by amylase enzymes.

5. Quantitatively transfer sample into a 50-ml beaker using 15 ml distilled water and add 100 µl thermostable α-amylase (14.4 U) into the suspension using a positive-displacement pipettor.

   Thermostable α-amylase is viscous and should be dispensed with a positive-displacement pipettor.

6. Mix suspension on a magnetic stirrer ≥15 min, then incubate 15 min in the boiling water bath, mixing every 5 min.

7. Cool suspension under continuous agitation and transfer to a 25-ml volumetric flask. Rinse beaker and stir-bar to ensure quantitative transfer. Bring to volume with water.

Hydrolyze starch to glucose

8. Transfer 1 ml diluted solution to a tube, add 50 µl amyloglucosidase (7 U) using a positive-displacement pipettor and 2 ml of 0.1 M sodium acetate buffer. Incubate 30 min in a 60°C water bath, mixing carefully every 5 min.

   Amyloglucosidase is viscous and should be dispensed with a positive-displacement pipettor.

9. Transfer mixture to a 25-ml volumetric flask, rinsing tube with water to ensure quantitative transfer, and dilute to volume with water.

   If a high-starch material (i.e., cornstarch) was used in the analysis, use a 100-ml volumetric flask.

10. Transfer duplicate 1-ml aliquots of the diluted mixture into two 10 × 100–mm glass test tubes.

11. Prepare a set of glucose standards by diluting 100 µg/ml glucose standard solution to 25, 50, and 75 µg/ml. Use water as a 0 µg/ml standard. Add 1 ml standard to duplicate tubes at each concentration of 0, 25, 50, 75, and 100 µg/ml glucose (10 tubes in all).

12. Prepare duplicate reagent blanks by adding 1 ml water to each of two tubes.

    Glucose controls and reagent blanks must be run with each set of samples.

Quantitate glucose

13. Add 1 ml GOPOD reagent to each tube (including glucose standards and reagent blanks), mix, and incubate 20 min in a 40°C water bath, mixing every 10 min.

14. Centrifuge 10 min at 3000 × *g*, at room temperature.

15. Transfer 1 ml supernatant from each tube into a disposable cuvette and measure the absorbance at 510 nm.

16. Develop a glucose standard curve by plotting glucose concentration versus absorbance.

17. Calculate true absorbance of the sample by subtracting blank absorbance from sample absorbance. Use the true sample absorbance and the glucose standard curve to determine µg of glucose in the sample.

18. Calculate total starch using the following equation:

    $$\% \text{ Starch} = \text{mg glucose} \times \text{DF} \times 100/W \times 162/180$$

    where mg glucose refers to the value calculated in step 17 (converted from µg to mg), DF is the dilution factor (25 × 25 = 625), 100/*W* is a factor used to express starch as a percentage of flour weight (*W*) in mg, and the fraction 162/180 adjusts from free glucose to anhydro glucose, as occurs in starch.

19. Calculate starch content on a dry weight basis (see Basic Protocol, step 20).

REAGENTS AND SOLUTIONS

*Use distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Glucose oxidase/peroxidase (GOPOD) reagent

Mix glucose determination reagent concentrate (Total Starch Assay Kit or Glucose Assay Kit; Megazyme International Ireland) with 1 liter of 1× phosphate buffer (see recipe). Store in aliquots, protected from light, for 3 months at 2° to 5°C or >1 year at −20°C.

Diluted GOPOD contains >12,000 U/liter glucose oxidase, >650 U/liter peroxidase, and 0.4 mM 4-aminoantipyrine.

MOPS (3-[N-morpholino]propanesulfonic acid) buffer, 50 mM

Add 11.55 g MOPS sodium salt (Sigma) to 900 ml water. Adjust pH to 7.0 with ~17 ml of 1 M (10%) HCl. Add 0.74 g $CaCl_2 \cdot 2H_2O$ and 0.2 g NaN_3. Adjust volume to 1 liter and store buffer up to 1 year at room temperature.

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves. It should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas. This chemical is added to buffers solely as a preservative and is not required if buffers are stored at <4°C.*

Phosphate buffer, 20×

13.6 g potassium dihydrogen orthophosphate
4.2 g sodium hydroxide
3 g 4-hydroxybenzoic acid
96 ml water
Adjust pH to 7.4 with 2 M HCl or 2 M NaOH
Bring to 100 ml
Add 0.4 g sodium azide
Store up to 3 years at <4°C
Before use, dilute 50 ml to 1 liter with water

The 20× phosphate buffer concentrate is supplied with the Megazyme kit (glucose reagent buffer).

Sodium acetate buffer, 0.1 M

Mix 1.16 ml glacial acetic acid and 1.64 g anhydrous $C_2H_3O_2Na$ in a final volume of 100 ml water. Adjust pH to 4.75 with 2 M HCl or 2 M NaOH. Store up to 3 years at <4°C.

Sodium acetate buffer, 200 mM

Add 11.8 ml glacial acetic acid (1.05 g/ml) to 900 ml water. Adjust pH to 4.5 with 1 M NaOH (~60 ml is required). Add 0.2 g NaN_3 and adjust volume to 1 liter. Store up to 3 years at <4°C.

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves. It should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas. This chemical is added to buffers solely as a preservative and is not required if buffers are stored at <4°C.*

Thermostable α-amylase, 100 U/ml

Dilute 1 ml thermostable α-amlyase stock (Total Starch Assay Kit, Megazyme International Ireland; 3000 U/ml) to 30 ml with 50 mM MOPS buffer (see recipe). Store diluted enzyme up to 3 years at −18°C between use.

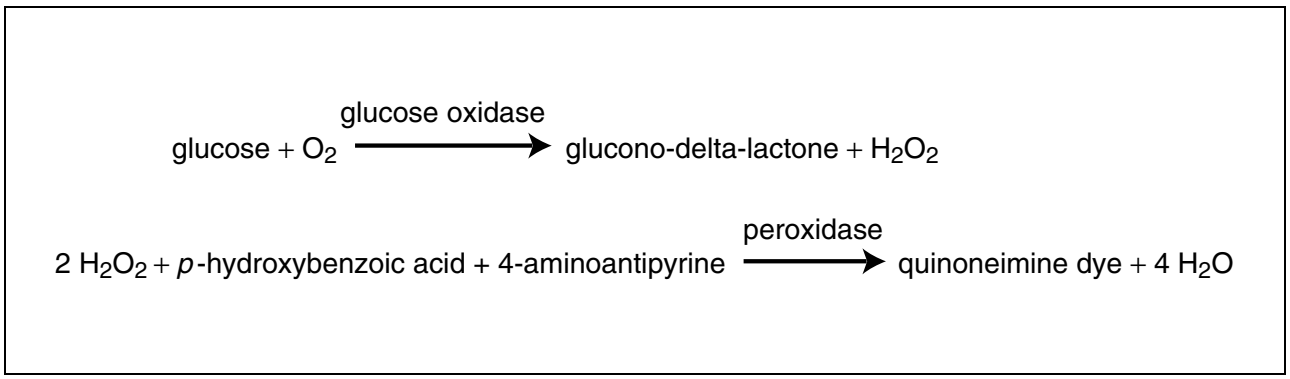

Figure E2.2.1 Principle of the colorimetric determination of glucose using GOPOD reagent.

COMMENTARY

Background Information

Numerous protocols of total starch determination have been developed over the last six decades; they are based on physical (polarimetric, specific gravity), chemical (acid hydrolytic or perchloric acid), and enzymatic methods. The choice of methodology for total starch determination in any application is highly dependent on the need for accuracy, precision, sensitivity, and rapidity. Physical and chemical methods, although rapid, proved to be of little value because of their lower specificity and sensitivity. Enzymatic methods, which use starch-specific amylase enzymes, although time consuming, have been shown to be highly accurate, precise, and sensitive. Enzymatic procedures vary in pretreatment steps (Karkalas, 1985) such as starch gelatinization, liquefaction, and dextrinization.

Enzyme hydrolysis of starch

Enzymatic starch determination is based on the selective conversion of starch in plant material to glucose by a combination of starch-specific amylase enzymes. The commonly used enzymes are thermostable α-amylase and amyloglucosidase. α-Amylase, an endoenzyme, dextrinizes starch by hydrolyzing α1-4 glycosidic linkages, yielding maltose units. High thermal stability of this enzyme enables its addition during boiling of starch in water. This leads to gelatinization (Biliaderis, 1991), liquefaction, and dextrinization (Robyt, 1984) in a single step. Amyloglucosidase, an exoenzyme, hydrolyzes both α1-4 as well as α1-6 glycosidic linkages of starch, yielding glucose units. At the end of hydrolysis, the glucose generated from starch is quantified, usually by a colorimetric technique that uses a reagent composed of glucose oxidase, peroxidase, *p*-hydroxybenzoic acid, and 4-aminoantipyrine (Figure E2.2.1). This reagent produces a pink color in the presence of glucose. Overestimation is unlikely as these enzymes are starch specific. However, the conversion of starch to glucose must be complete in order to avoid underestimation. Factors that influence the conversion of starch to glucose are: (1) the presence of native starch crystals; (2) other grain components that surround the starch granules; and (3) the presence of resistant starch. These factors are discussed below.

Factors that influence the conversion of starch to glucose

Presence of native starch crystals. In plant materials, starch exists as semicrystalline, granular entities. Starch molecules, both amylose and amylopectin, with their many intermolecular hydrogen bonds, give structure to the starch granule (Zobel, 1988). The crystalline regions formed by this intermolecular hydrogen bonding are somewhat resistant to enzyme hydrolysis and need to be broken prior to enzyme treatments.

Grain components that surround starch granules. Starch granules are surrounded by other grain components, such as proteins, cellulose, β-glucans, and lipids, which can hinder the access of enzymes to starch. Therefore, in order to ensure quantitative conversion of starch to glucose, plant material or sample is usually dry ground and gelatinized by heating in water (Biliaderis, 1991) prior to enzyme treatment. Dry grinding breaks open the surrounding grain components, while gelatinization ensures partial solubilization of starch in water and opening of the granular or crystalline structure by breaking the intermolecular hydrogen bonds. Addition of thermostable α-amylase during the gelatinization step ensures total solubilization of starch. Differences between

the protocols in this unit are mainly in this first phase of starch solubilization.

Presence of resistant starch. Most foods are heat processed prior to consumption. Although heat processing increases the availability of starch to enzymes, a fraction of starch remains resistant to amylase hydrolysis in the human gastrointestinal tract; this fraction is called resistant starch (RS). Englyst and Cummings (1987) classified RS into three groups: RS1 is physically inaccessible starch, RS2 is crystalline regions of native starch granules and retrograded amylopectin, and RS3 is retrograded amylose. The RS1 and RS2 are slowly but completely digested with appropriate preprocessing of foods, but RS3 totally resists digestion. Although RS3 exists in small quantities in native grain flours, studies have shown that heat processing enhances its content. Amylose crystallization during retrogradation of starch is responsible for RS3 formation (Berry, 1986; Bjorck et al., 1987; Sievert and Pomeranz, 1989; Sievert et al., 1991; Leloup et al., 1992). Miles et al. (1985) reported that amylose crystallization occurs through chain elongation, as double helices form between amylose molecules near the terminal regions of the chains, and chain folding, as the elongated amylose chains fold and facilitate helix-helix aggregation or packing by formation of interhelical hydrogen bonds. The intimate packing of starch double helices results in crystal formation (Imberty et al., 1991), which resists the diffusion of starch hydrolyzing enzymes into the region and their binding to starch chains, making it resistant to enzyme hydrolysis.

Critical Parameters

Particle size reduction of samples by milling should be performed uniformly. The uniformity can be ensured by sieving the milled samples through a 0.5-mm screen. The preexisting glucose and maltodextrins in a sample can lead to overestimation. Sample blanks should be run in parallel in order to avoid overestimation. Alternatively, analysis of the sample blank can be avoided by preextracting samples with 80% ethanol.

With each new batch of GOPOD reagent, the time for maximum color formation with 100 μg of glucose standard should be checked (usually 15 min). An expected starch content <65% is considered low.

Anticipated Results

A number of analyses carried out by the author revealed that both Basic and Alternate Protocols are equally good for starch determination. Some of the expected values (all w/w; dry weight) are presented for grains (wheat, 55% to 60%; corn, 65% to 72%; barley, 55% to 63%; oat, 53% to 62%; field pea, 55% to 63%; and lentil, 56% to 60%) and processed products (wheat bran, 35% to 45%; wheat pasta, 80% to 85%; all-purpose wheat flour, 75% to 80%; and pure corn starch, 94% to 96%). Interlaboratory evaluation (McCleary et al., 1994) of the Megazyme procedure has shown that a standard error of ±2% is routinely achieved.

Time Considerations

There have been a number of research efforts to enhance the rapidity of the enzymatic method, but still the minimum time requirement remains high. The author's laboratory evaluation indicated that the Basic Protocol (Megazyme assay) can handle 20 samples in 3 hr and 15 min, whereas the Alternate Protocol (Holm method) will take 3 hr and 45 min for the same number of samples.

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Contributed by Thava Vasanthan
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Determination of Total Amylose Content of Starch

BASIC PROTOCOL

Starches from various sources contain different amounts of amylose and amylopectin, which influence physicochemical properties such as gelatinization, retrogradation, water absorption, and paste viscosity. For those reasons, the amylose content must be determined accurately. Previous studies have shown that colorimetric methods (based on amylose-iodine complex formation) are influenced by amylopectin chain length (which is known to vary in starches from different botanical sources). For example, the outer branches of potato amylopectin, which are longer than those of cereal starches, can bind iodine and cause an overestimation of the amylose content if not taken into consideration. This Basic Protocol takes into account the iodine affinity of amylopectin and involves the following steps (1) starch defatting; (2) dispersion of starch in dimethylsulfoxide (DMSO); (3) determination of the absorbance at 600 nm of the dispersed starch; (4) preparation of a standard curve over the range 0% to 100% amylose, using mixtures of pure amylose and amylopectin; and (5) calculation of total amylose content from the standard curve. In this protocol, potato is the source of amylose and amylopectin.

Materials

Starch sample
75% (v/v) *n*-propanol in water
Pure potato amylose type III and amylopectin (Sigma)
90% (v/v) dimethylsulfoxide (DMSO) in H_2O
Iodine solution (see recipe)

Cellulose extraction thimble
Cotton wool plug
Heating mantle
Soxhlet apparatus
30°C forced air oven
Round-bottom screw-cap tubes with a Teflon-faced rubber liner in caps
Vortex mixer
85°C water bath
50-ml volumetric flask
Quartz cell (1-cm path-length)
UV/visible spectrophotometer

Defat starch sample

1. Weigh accurately 5.0 g of starch into a cellulose extraction thimble and cover the mouth with a cotton wool plug.
2. Extract the lipids with 120 ml 75% *n*-propanol at 85°C with a heating mantle for 7 hr in a Soxhlet extractor.

   Lipid-complexed amylose chains interfere with amylose-iodine (I_3^- and I_5^-) complex formation. Therefore, extraction of complexed lipids with 75% n-propanol is necessary prior to determination of total amylose content.

3. Air dry the thimble containing the lipid-free starch for 12 hr.
4. Remove the lipid-free starch from the thimble and oven dry 24 hr, 30°C.

Contributed by R. Hoover and W.S. Ratnayake
Current Protocols in Food Analytical Chemistry (2001) E2.3.1-E2.3.5

Disperse lipid-free starch (Solution I)

5. Weigh 20 mg of lipid-free starch (correct to 0.1 mg) into a round bottom screw-cap tube fitted with a Teflon-faced rubber liner in the cap.
6. Prepare a series of mixtures of pure potato amylose and amylopectin (0%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, and 100% amylose). Weigh 20 mg of each into round-bottom tubes with caps.
7. Add 8 ml 90% DMSO to each round-bottom tube and mix vigorously for 2 min using a vortex mixer.
8. Heat the tubes in a water bath at 85°C for 15 min with intermittent mixing.
9. Allow the tubes to cool to room temperature (~45 min).
10. Check for the presence of clear gels in the cooled, dispersed solution.

    If a gel is detected, repeat steps 5 to 9 with more vigorous mixing at shorter time intervals in step 7.

11. Dilute the sample to 25 ml with water in a volumetric flask.

Determine absorbance of dispersed starch solution

12. Add 1.0 ml of the diluted solution (Solution I) and 40 ml of distilled water into a 50-ml volumetric flask.

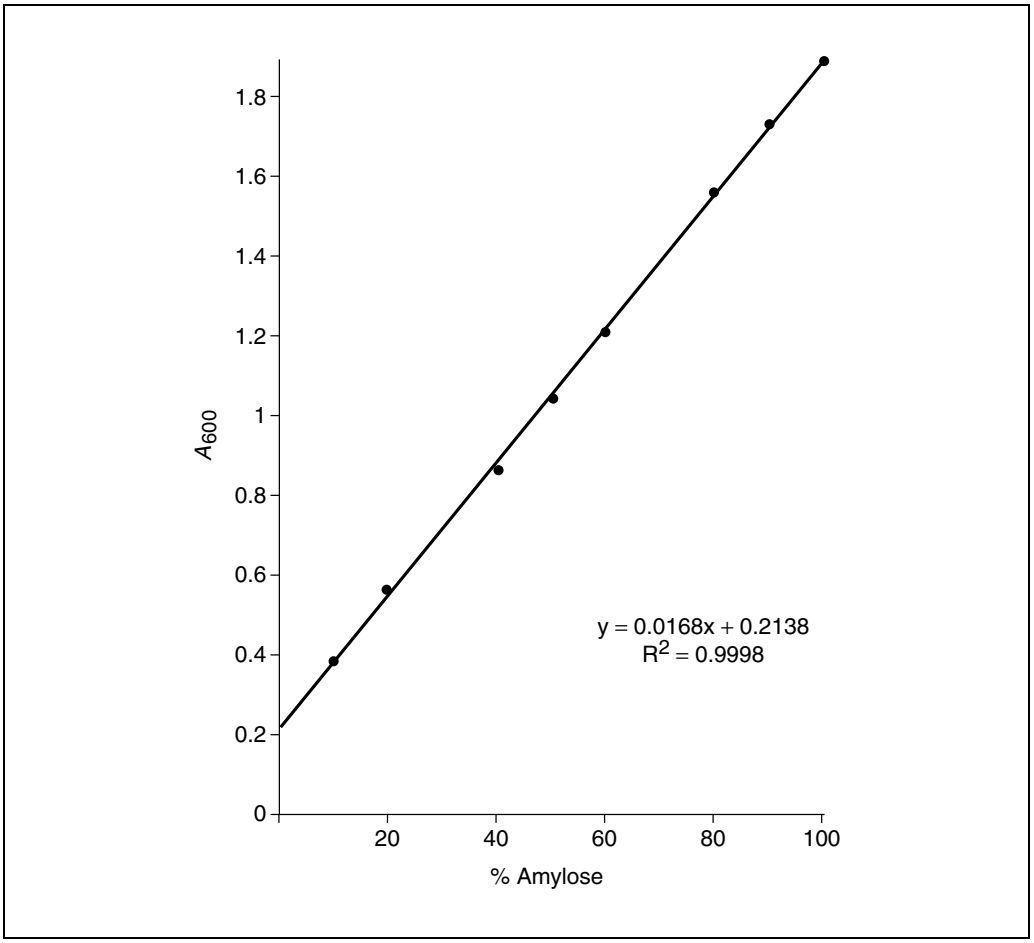

Figure E2.3.1 Plot of absorbance at 600 nm against percentage amylose (w/w) for mixtures of potato amylose and amylopectin with iodine. The absorbance of 0% amylose is due to the I_2 affinity of the long outer branches of amylopectin.

13. Add 5 ml of iodine solution and mix vigorously. Then, adjust the volume to 50 ml with distilled water and mix vigorously. Allow color to develop for 15 min.
14. Mix the contents of the flask by hand.
15. Measure the absorbance of the sample and each of the standard mixtures at 600 nm against a reagent blank as the reference.

The reagent blank contains all reagents in the same amounts without the sample containing starch.

Prepare standard curve for mixtures of amylose and amylopectin

16. Plot a standard curve (Fig. E2.3.1) for mixtures of pure potato amylose and amylopectin.
17. Determine the regression equation for the standard curve and use this equation to calculate the total amylose content of the sample.

The regression equation for the standard curve shown in Figure E2.3.1 is y = 0.0168x + 0.2138 (R^2 = 0.9998); where x = % amylose and y = absorbance at 600 nm.

$$\frac{\text{absorbance} - 0.2138}{0.0168} = \%\ \text{amylose}$$

The standard deviations of all the data points in Figure E2.3.1 were less than 0.01 (not shown), and the data points are means of three individual determinations against a reagent blank.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Iodine solution

Prepare a 0.0025 M I_2/0.0065 M KI mixture. For a 100-ml solution: Dissolve 0.1079 g KI in 5 ml of water in a 100-ml volumetric flask. Add 0.0315 g I_2, shake well (by hand) until all I_2 is dissolved in KI. Adjust the volume to 100 ml. The solution should be stored in a dark bottle, 4°C, until used.

COMMENTARY

Background Information

The formation of a helical complex between amylose and iodine gives rise to the typical deep blue color of starch dispersions stained with iodine and forms the basis for quantitative determination of amylose content. In I_2/KI solutions, polyiodide ions such as I_3^- and/or I_5^- (Teitelbaum et al., 1978, McGrance et al., 1998) interact with amylose forming single left-handed V-type helices. The helix consists of six anhydrous glucose residues per turn with a pitch of 0.8 nm and a hydrophobic helical cavity diameter of 0.5 nm. McGrance et al. (1998) have shown that an I_5^- is present within almost every turn of the amylose helix, parallel to its long axis. Sen et al. (1997) have shown that the iodine affinity of amylose varies between 17 and 22 g I_2 per 100 g amylose in maize, according to the genotype, while the affinity of amylopectin for iodine is only 1.05 to 1.25 g I_2 per 100 g amylopectin. Amylopectin and the iodine complex exhibits a purple color with λ_{max} at 530 nm. Morrison and Karkalas (1990) have shown that there are atypical types of amylopectin, (1) high molecular weight amylopectin with A and B chains 5 to 15 glucose residues longer than normal; (2) amorphous amylopectin with similarly extended A and B chains that elute from GPC columns with amylose; and (3) normal amylopectin which contain very long chains (CL 85-180) with infrequent branching. Atypical types of amylopectin bind more iodine and give higher λ_{max} than normal amylopectin, so that they give an in-

Table E2.3.1 Determination of Total Amylose Content

Starch source[a]	Total amylose content (%)[b]
Potato	24.03 ± 0.04
Field pea	43.73 ± 0.03
Oat	23.07 ± 0.04

[a]Starches were defatted prior to amylose estimation using the method outlined in the Basic Protocol.

[b]Total amylose content was determined using the Basic Protocol. The values represent the means ± standard deviations of three determinations.

flated value in the iodometric determination of amylose. Banks and Greenwood (1975) have shown that atypical types (1) and (2) amylopectins occur in several legume and tuber starches; and type (3) has been reported in sweet potato starches (Takeda et al., 1986). The authors have used the methods cited in the literature (McCready and Hassid, 1943; Williams et al., 1970; Juliano, 1971; Morrison and Laignelet, 1983; Knutson, 1986; Yun and Matheson, 1990; Sen at al., 1997; Mohammadkhani et al., 1999) for the iodometric estimation of amylose content of cereal, tuber, and legume starches. The authors' experience has shown that none of the above methods yield reproducible results. The method reported in this unit for total amylose content is a modification of the procedure of McGrance et al. (1998). The authors have found the 75% *n*-propanol solvent system used to defat the starch to be more efficient than the water-saturated butanol used by McGrance et al. The iodine solution has also been optimized. This method is reproducible, can be applied to low and very high amylose containing starches, and also takes into account the contribution from amylopectin to λ_{max}. Furthermore, the defatting step in this procedure minimizes interference of amylose-lipid complexes in amylose determination.

Critical Parameters and Troubleshooting

The defatted starch should not be removed from the thimble until contents have been air-dried for at least 12 hr. Removal prior to this would result in contamination of starch with cellulose from the thimble. The dried defatted starch should be in the form of a free flowing powder. The presence of aggregated starch clumps would result in the formation of insoluble gels during step 9.

In step 9, a clear gel may develop during the determination of high amylose containing starches (this can be confirmed by centrifuging the dispersed sample at 10,000 × *g* for 15 min. The presence of a gel would be indicated by the formation of a precipitate at the bottom of the centrifuge tube). If a gel is detected, repeat steps 5 to 9 with more vigorous mixing at shorter time intervals during the heating cycle in step 8.

A quartz cell (1-cm path length) must be used for measuring absorbance. The use of disposable plastic cells will introduce a significant experimental error due to the variations in the absorbances resulting in non-reproducible results.

The color development (step 13) should be conducted within the temperature range 23° to 25°C. Temperatures outside this range will significantly alter the absorbance.

Anticipated Results

Table E2.3.1 illustrates the total amylose content of field pea, oat, and potato starches. The values (which are means of triplicate measurements) are reproducible and the method can be used for all types of starches. Very high amylose containing starches (i.e., >50% amylose) may not yield consistent values if suitable precautions (see Critical Parameters and Troubleshooting) are not taken during step 9. The method can easily accommodate small samples for microdeterminations, if necessary.

Time Considerations

For analysis of amylose content of oat, field pea, and potato starches (in triplicate) may take ~53 hr.

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Yun, S.H. and Matheson, N.K. 1990. Structural changes during development in the amylose and amylopectin fraction separated by precipitation with concanavalin A of starches from maize genotypes. *Carbohyd. Res.* 270:85-101.

Key Reference

McGrance et al., 1998. See above.

Gives detailed experimental information about the selection of the wavelength and the calculations for the determination of the amount of iodine that should be used.

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Isolation of Plant Cell Walls and Fractionation of Cell Wall Polysaccharides

Plant foods are an important component of the diet of humans. Since the cell wall makes up a large component of the dry weight of plant tissues, and because in a normal diet most of the dietary fiber comes from plant cell walls, it follows that there is a need to know the composition of the cell walls. Moreover, the cell wall contributes (along with turgor pressure and the cell contents) to the texture of plant foods, and since humans consider the texture of foods an important sensory attribute, again, understanding wall composition is important.

While for some plant species the cell wall composition has been studied, the actual three-dimensional organization of the polysaccharides in the cell wall has not been fully elucidated. Moreover, it appears that while some aspects are constant for all walls (i.e., the presence of similar types of polysaccharides), the relative proportion and fine structure of the polysaccharides may be different, and thus the way they are organized in the wall may be different. Determination of the composition of polysaccharides in cell walls is commonly done by chemical analytical methods, but prior to determining the composition or structure of the polysaccharides in cell walls, the walls must first be isolated from the plant tissues and separated from the intracellular material.

The first step in isolating cell walls is to examine the plant material microscopically (see Basic Protocol 1) to determine the types of cells in the tissues and to detect the presence of starch. If starch is present in large amounts, it must be actively removed or else there may be more starch than cell walls, and the carbohydrate analyses will be meaningless. Some plants contain large amounts of starch in which almost every cell is packed full of granules (e.g., potato), while others contain small amounts of starch in which some of the cells contain granules (e.g., apples and unripe tomatoes), and still other plant tissues may contain very little or no starch (e.g., onions and pineapple). Even among the same plants, all material must be checked because there will be differences among varieties, stages of ripeness, and parts of the plants used.

Table E3.1.1 Summary of Protocols in this Unit

Protocol	Description
Basic Protocol 1	Microscopic examination of plant material
Basic Protocol 2	Isolation of cell walls from plant tissues that do not contain starch using phenol-HEPES
Alternate Protocol 1	Isolation of cell walls from plant tissues that do not contain starch using HEPES buffer
Basic Protocol 3	Isolation of cell walls from plant tissues that contain starch using phenol-HEPES
Alternate Protocol 2	Isolation of cell walls from plant tissues that contain large amounts starch using HEPES buffer
Alternate Protocol 3	Isolation of cell walls from plant tissues that contain small amounts of starch using HEPES buffer
Basic Protocol 4	Fractionation of plant cell wall polysaccharides
Support Protocol 1	Preparation and use of dialysis tubing

Contributed by Laurence D. Melton and Bronwen G. Smith
Current Protocols in Food Analytical Chemistry (2001) E3.1.1-E3.1.23

The next step is to select a protocol for isolating cell walls that suits the type of material to be investigated and the reasons for doing the research (see Critical Parameters). Two basic protocols are described, one for plant tissue that does not contain starch (see Basic Protocol 2) and one for plant tissue that does contain starch (see Basic Protocol 3), as well as three alternate protocols that can be used and modified to suit (see Commentary).

The final step is fractionation of cell wall polysaccharides, which is a sequential chemical extraction of polysaccharides from the walls (see Basic Protocol 4). Table E3.1.1 provides a more detailed description of the protocols presented in this unit.

BASIC PROTOCOL 1

MICROSCOPIC EXAMINATION OF PLANT MATERIAL

The objective of microscopic examination is to determine the cell types present in the tissues of the plant material to be studied so that a homogeneous preparation of walls from one type of cell can be isolated. The simple techniques described in this protocol will allow investigators to distinguish between cells with primary unlignified walls and those with secondary lignified walls. Furthermore, any starch present in the cells will be detected. In brief, hand-cut sections are prepared from the plant material and examined by light microscopy after staining with toluidine blue O, phloroglucinol-HCl solution, and iodine in potassium iodide solution.

Examination of sections from parts of the tissue from which cell walls are to be isolated will determine the types of cells present. Most of the tissues of foods that are commonly consumed are parenchyma cells. Parenchyma cells have unlignified cell walls, stain pink-purple with toluidine blue, and are not stained with phloroglucinol-HCl solution; starch may be present or absent. Sections which contain vascular tissue (xylem) stain red with phloroglucinol-HCl and green with toluidine blue due to the lignin in the walls. Xylem cells have secondary cell walls and have a different polysaccharide composition. Thus, knowing the types of cells in the tissue to be isolated is useful in interpreting the polysaccharide composition.

Examination of the tissue with iodine in potassium iodide solution will determine the appropriate cell-wall isolation protocol. If most of the cells are packed full of starch granules, then it is suggested the experimenter choose between Basic Protocol 3 and Alternate Protocol 2. If starch granules are present in some of the cells then it is suggested the experimenter choose between Basic Protocol 3 and Alternate Protocol 3. If only a few very small granules are present it is suggested the experimenter choose between Alternate Protocol 3 or Basic Protocol 2.

Materials

Plant material, fresh and free from bruises and pathogens
Ethanol
Carrot (for leafy materials; optional)
Toluidine blue O (see recipe)
Phloroglucinol-HCl solution (see recipe)
Nail varnish or other sealant
Iodine in potassium iodide solution (see recipe)

Scalpel
Double-edged razor blades
Watch glass
Artists brush, small
Compound light microscope with appropriate glass microscope slides and coverslips

1a. *For most plant material:* Trim the plant material into a conical shape ~30 × 20–mm at base with a scalpel. Wipe razor blade carefully with ethanol to remove any grease. Wet the blade and the plant material well with water. Rest elbows on the bench and hold the material between the thumb and index finger. Hold the razor blade in the other hand. Cut thin sections (1 to 3 cells thick) by moving the blade in a diagonal

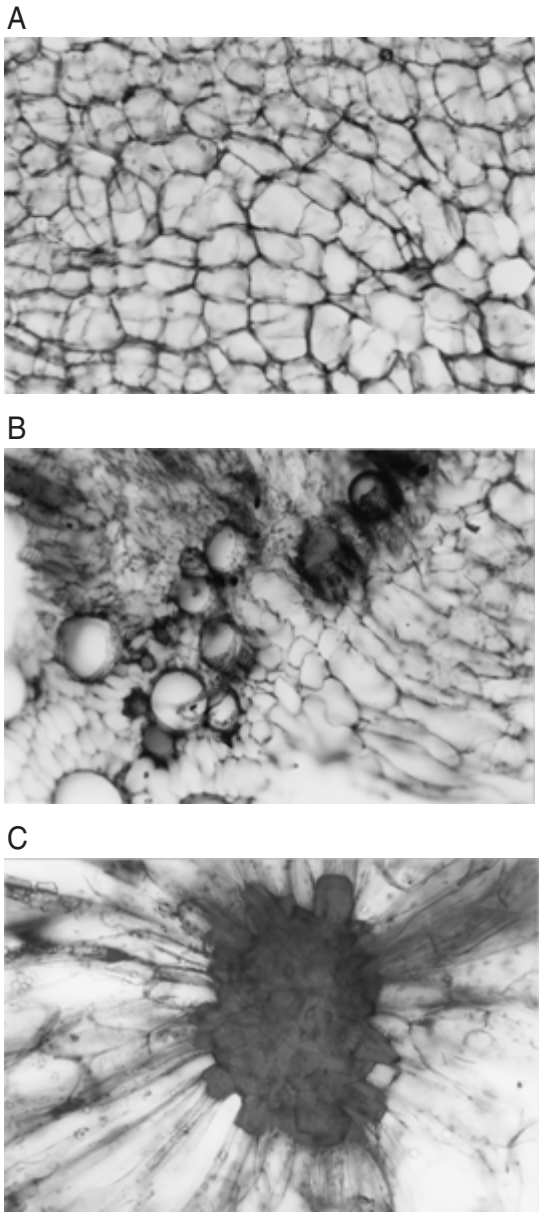

Figure E3.1.1 (**A**) Hand-cut section from fresh carrot tissue stained with toluidine blue O. Cell walls of the parenchyma tissue are stained pink-purple, indicating they are unlignified. (**B**) Hand-cut section from fresh carrot tissue stained with toluidine blue O. Cell walls of the parenchyma tissue are stained pink-purple indicating they are unlignified and cell walls of the xylem tracheary elements (appear as circles) are stained blue-green indicating they are lignified. (**C**) Hand-cut section from fresh pear tissue stained with toluidine blue O. Cell walls of the parenchyma tissue are stained pink-purple indicating they are unlignified and walls of the sclereid cells (stone cells; center) are stained blue-green indicating they are lignified. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

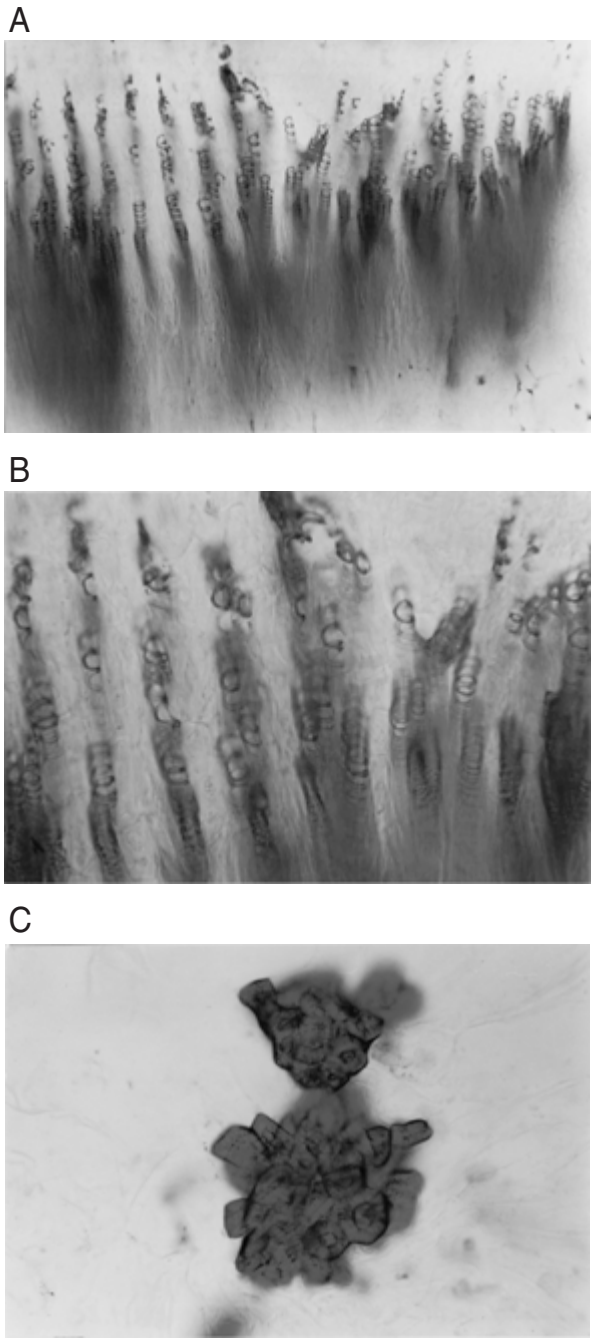

Figure E3.1.2 (**A**) Hand-cut section from fresh apple tissue stained with phloroglucinol/HCl. Cell walls of the xylem tracheary elements (spirals) are stained red indicating they are lignified. (**B**) Increased magnification of A. (**C**) Hand-cut section from fresh pear tissue stained with phloroglucinol/HCl solution. Walls of the sclereid cells (stone cells; center) are stained red indicating they are lignified. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

direction across the tip. Wash the sections that accumulate in the film of water on the blade into a watch glass. Select the thinnest sections for staining using a small artist's brush.

While parts of the section may be too thick for observation the edges of the section will probably be just a few cells thick and allow the necessary observations. The best sections are made with new blades. Only a few sections should be cut on each part of the blade.

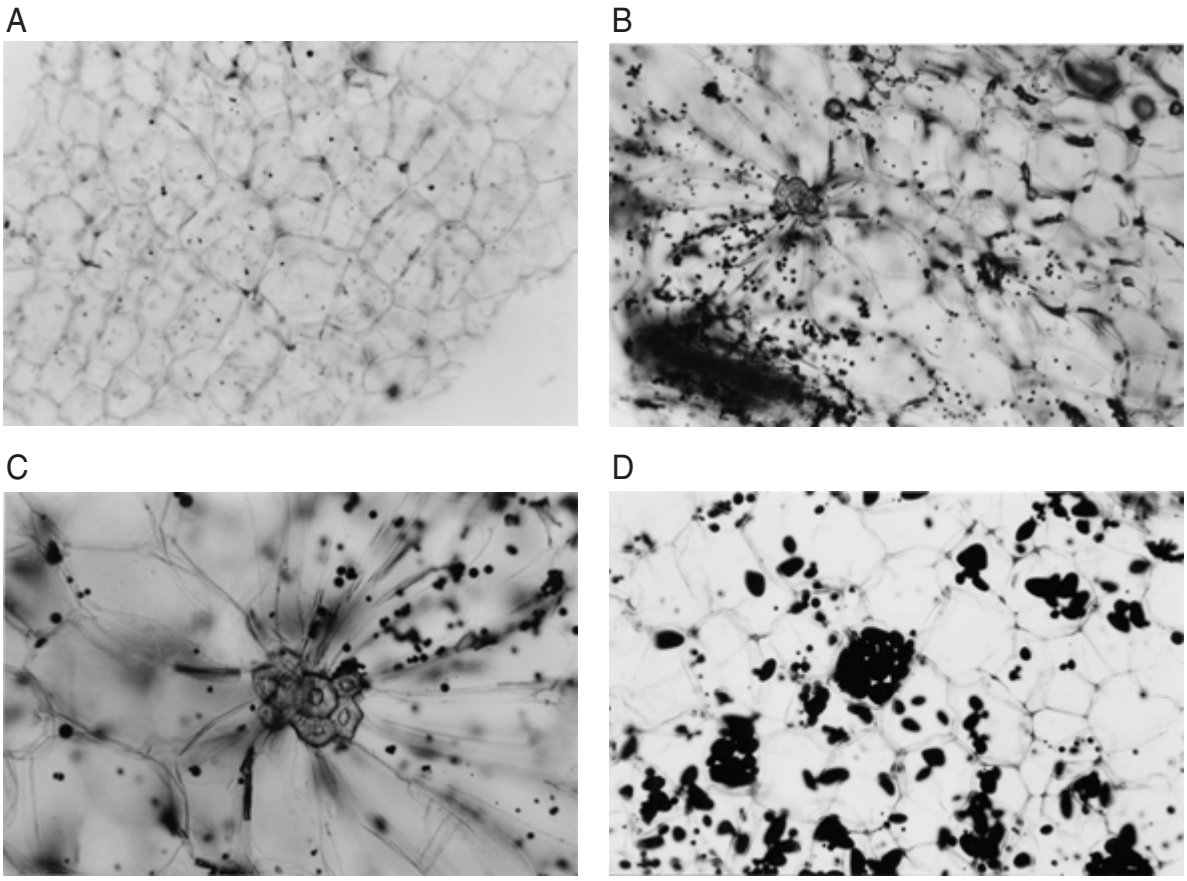

Figure E3.1.3 (**A**) Hand-cut section from fresh apple tissue stained with iodine in potassium iodide solution showing a small amount of small starch granules (black spots). (**B**) Hand-cut section from fresh pear tissue stained with iodine in potassium iodide solution showing a large amount of small starch granules. (**C**) Increased magnification of B. (**D**) Hand-cut section from fresh potato tissue stained with iodine in potassium iodide solution showing a large amount of large starch granules. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

A good description of hand sectioning is given in O'Brien and McCully (1981).

1b. *For leafy material:* Prepare a cone of carrot and make a slice part way through from the top. Insert a piece of leaf. Make the sections as described above, discarding the pieces of carrot.

2. Place section in a watch glass and add a few drops of toluidine blue. Stain for 1 min and then wash with water for 1 min. Mount in water on a glass microscope slide. View with a compound light microscope. Compare against unstained control sections mounted in buffer as the stain.

Toluidine blue O stains walls containing lignin blue-green and unlignified walls pink-purple. See Figure E3.1.1, panels A to C.

3. Place another section on a new slide and add one or two drops of the phloroglucinol-HCl solution. Place a coverslip over the section and seal the edges with nail varnish or other sealant. View immediately as color fades quickly. Compare against control sections mounted in 1:2 95% ethanol:HCl.

CAUTION: *HCl is very corrosive; take special care to prevent contact with skin and microscope objectives. Also take care not to contact microscope objectives with nail varnish. See APPENDIX 2B for more information.*

Walls containing lignin stain red (Harris et al., 1980). See Figure E3.1.2, panels A to C.

4. Place another section on a new slide and add one or two drops of the iodine in potassium iodide solution. Place cover slip over the section and view with a compound light microscope. Compare against unstained control sections mounted in water. See Figure E3.1.3, panels A to D.

   Starch granules stain blue-black.

BASIC PROTOCOL 2

ISOLATION OF CELL WALLS FROM PLANT TISSUES THAT DO NOT CONTAIN STARCH USING PHENOL-HEPES

The following basic protocol is a procedure for isolating cell walls from tissues that contain very little or no starch. The purpose of the cell wall isolation procedure is to obtain a preparation of cell walls that is virtually free from contamination by cytoplasmic components of the cells (e.g., membranes, nuclear material, enzymes, and starch). This is achieved through a series of steps that involve methods of breaking cells open and washing away the cell contents. The washes are retained to determine the composition of polysaccharides lost from the cell walls due to their solubility in aqueous solutions. An important consideration in cell wall isolation is that endogenous cell wall enzymes may degrade the polysaccharides in the wall during the procedure. The protocol described below includes phenol in the extraction buffer to inactivate enzymes and solubilize proteins; however, phenol is very corrosive and requires special handling. Furthermore, its effect on the polysaccharides in the wall is unclear. An alternative is to use HEPES buffer (see Alternate Protocol 1).

Materials

50 g plant material, fresh and free from bruises and pathogens
Liquid nitrogen
Phenol-HEPES extraction mixture (see recipe)
Ponceau 2R (see recipe)
50 mM HEPES buffer, pH 6.7 (see recipe)
50 mM HEPES buffer, pH 6.7 (see recipe) containing 20% ethanol
Acetone
Dry ice

Mortar and pestle, dried and chilled with liquid nitrogen
Thick glass stirring rod
Compound light microscope and glass slides
Ultra-Turrax (IKA Werke) or Polytron homogenizer (Kinematica)
Sorvall RC-28S superspeed centrifuge (or equivalent), 250-ml screw-cap high-density polyethylene (HDPE) centrifuge bottles, and appropriate rotor
Dialysis tubing (see Support Protocol)
200 × 200 × 50–mm stainless steel container
50-ml round-bottom flask
Freeze dryer (VirTis)
Desiccant (e.g., silica gel)

CAUTION: Phenol is corrosive. Gloves and safety goggles should be worn when handling. See *APPENDIX 2B* for more information.

Homogenize plant tissue

1. Select plant material for cell wall isolation based on microscopic examination (see Basic Protocol 1). Take 50 g of tissue (in duplicate or preferably triplicate).

2. Quickly cut the tissue into small pieces (e.g., 1 cm^3) and place each piece into liquid nitrogen as it is produced.

   CAUTION: *Wear protective gloves and safety goggles to prevent injury from liquid nitrogen. See APPENDIX 2B for more information.*

3. Place frozen pieces of tissue into a mortar cooled with liquid nitrogen. Grind with a pestle to form a fine powder. Keep adding liquid nitrogen to the tissue to prevent thawing. Grind small amounts of tissue at a time, placing the ground material in a beaker and keeping it frozen by adding liquid nitrogen.

4. Place the frozen powder in a beaker in alternating layers with 100 ml of phenol-HEPES extraction mixture.

5. Prod the mixture with a thick glass stirring rod to assist thawing and to mix the layers together to form a thick slurry.

   It is important that all of the frozen tissue be mixed thoroughly with the phenol-HEPES extraction mixture.

6. Homogenize the slurry using an Ultra-Turrax or Polytron homogenizer, in 20-sec bursts for 6 min, at 24,000 rpm.

   The mixture is initially a frozen slurry. The homogenization procedure is performed to break open cells and at the same time ensure thorough mixing of the phenol solution and the plant material to destroy the enzymes. As a consequence of prodding with the glass rod and homogenization, the mixture does warm up slightly to ~4°C. When buffer and DTT are used as the homogenizing media instead of phenol (see Alternate Protocols 1, 2, and 3), the main protection against enzymatic action is the cold temperature; therefore, in those situations, the extra precaution of performing the procedure on ice should be exercised.

7. Check the extent of cell wall breakage by placing a drop of the homogenate on a glass slide, adding a drop of Ponceau 2R, and viewing with a compound light microscope.

   Ponceau 2R stains proteins red and thus unbroken cells can be detected from the staining of their cytoplasmic contents (Harris, 1983). It is likely at this stage that clusters of unbroken cells are present together with some broken cells. Further homogenization treatments can be tried, or it may be necessary to use a different device (e.g., a ring grinder or rock grinder; Rocklabs) to rupture the remaining cells after an initial wash in buffer. (For details on rock grinders and ring grinders see http://www.rocklabs.com; devices such as these are used by geologists.)

8. Transfer the mixture to a 250-ml screw-capped high-density polyethylene (HDPE) centrifuge bottle, using 50 mM HEPES buffer, pH 6.7, to wash remaining residue from the beaker into the centrifuge bottle. Centrifuge at 8600 × *g* for 20 min, room temperature.

   The authors currently use a Sorvall RC-28S superspeed centrifuge and the appropriate rotor; however, experimenters should use whatever is available. The most important thing is to achieve separation of the liquid and solid phases.

   If the separation of solid and liquid is incomplete, centrifuge at a higher speed.

9. Remove the supernatant with a large pipet. Add the phenol-HEPES-soluble fraction to dialysis tubing, and dialyze against running tap water for 2 days followed by dialysis against three changes of distilled water (e.g., 5 liters) of 8 hr each.

10. Resuspend the pellet in 50 ml of 50 mM HEPES buffer, pH 6.7/20% ethanol. Centrifuge as described in step 8. Remove the supernatant as before and retain at 4°C.

11. Keep the cell wall pellet in the plastic centrifuge bottle to minimize losses. Resuspend the pellet in 100 ml of 50 mM HEPES buffer, pH 6.7/20% ethanol. Stir for 6 hr at room temperature. Centrifuge at 9800 × *g* for 20 min at room temperature. Remove the supernatant as before and retain at 4°C.

12. Resuspend the pellet in 100 ml of 50 mM HEPES buffer, pH 6.7 (no ethanol). Stir for 18 hr at room temperature. Centrifuge at 9800 × *g* for 20 min at room temperature. Store pellet at 4°C for use in step 17. Remove the supernatant as before and retain at 4°C.

Freeze dry water-soluble fraction

13. Combine all the supernatants from steps 10, 11, and 12. Dialyze the fraction against running tap water overnight and then against three changes of distilled water (e.g., 5 liters) for 8 hr each.

    This combined fraction is the water-soluble fraction.

14. Prepare a small bath (e.g., 200 × 200 × 50–mm made of stainless steel) containing dry ice and acetone. Chill a 50-ml round-bottom flask in the bath. Slowly add small volumes of the fraction (a few milliliters at a time) to the flask, gently rotating it in the bath to shell coat the sample.

    CAUTION: *Use gloves and safety glasses for this step.*

    Gradually, the inside of the flask will become coated with the frozen fraction. Do not overfill the flasks.

    The authors usually freeze-dry samples for convenience and analyze later with the cell walls and cell-wall fractions.

15. Place the small flasks into freeze-dryer flasks and attach to the ports on the manifold of the freeze dryer.

16. Remove freeze-dried material from the sides of the flasks using a spatula. Gently scrape off any other material that is adhering. Weigh and analyze (*UNIT E3.2* and *UNITS E3.3 & E3.4)*.

Examine cell wall isolate

17. Examine a small drop of the pellet from step 12 microscopically using Ponceau 2R to confirm that all cells have broken open (see step 7).

    This pellet is the cell wall isolate.

18. Place the isolated cell walls in a clean vial. Record total wet weight and store at 4°C for chemical fractionation as described in Basic Protocol 4.

    Chemical fractionation should be started within 12 to 15 hr of isolation.

19. For analyses of whole cell walls, remove a small aliquot, record weight, freeze dry, and weigh. Proceed to analyses (e.g., *UNITS E3.2, E3.3, & E3.4*).

20. To estimate the recovery of cell walls as dry weight, remove a small aliquot, record weight, freeze dry and then further dry to a constant weight over desiccant (e.g., silica gel).

ISOLATION OF CELL WALLS FROM PLANT TISSUES THAT DO NOT CONTAIN STARCH USING HEPES BUFFER

ALTERNATE PROTOCOL 1

In the protocol for isolating cell walls, phenol was included in the isolating medium to destroy enzymes (see Basic Protocol 2); however, as phenol is corrosive and requires special handling procedures, the following alternative isolation protocol is described. The authors emphasize that the main protections against enzyme activity in this alternative protocol are low temperatures and the experimenter working very quickly. The buffer used is 20 mM HEPES buffer, pH 6.7, containing 10 mM dithiothreitol (DTT). HEPES is preferred because it does not chelate calcium ions associated with pectic polysaccharides (Fry, 1988); moreover, HEPES buffer resists a change in pH with lowering of temperature. The reducing agent DTT is added to reduce the activity of polyphenol oxidases. All procedures are performed quickly and at ≤4°C to minimize the activity of wall-degrading enzymes. A trial isolation of each different plant material is necessary to determine the isolation parameters, such as duration of homogenization as the times given in the protocol are averages (see Critical Parameters).

Additional Materials *(also see Basic Protocol 2)*

Plant material, fresh and free from bruises and pathogens
20 mM HEPES buffer, pH 6.7 (see recipe) containing 10 mM DTT

11-μm nylon mesh (Nybolt)
Buchner funnels
Ring grinder with a zirconia head (e.g., Bench Top Ring Mill; Rocklabs) or equivalent

1. Homogenize plant tissue as described elsewhere (see Basic Protocol 2, steps 1 to 7), substituting 20 mM HEPES, pH 6.7 buffer containing 10 mM DTT for phenol-HEPES extraction mixture (step 4) and keeping the slurry at 4°C on ice during the homogenization (step 6).

2. Wash the preparation by first pouring the homogenate onto the 11-μm nylon mesh in a Buchner funnel placed over a sturdy flask, and then adding 2 × 50-ml applications of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C. Collect the filtrate and keep at 4°C.

   If this filtration step does not proceed rapidly, it may be because the pores of the mesh have become blocked. Resolve by either trying a larger mesh size (e.g., 54 μm) or assisting filtration by gently aspirating the homogenate with a Pasteur pipet.

3. Remove the cell wall material from the mesh and place in a beaker on ice. Grind aliquots of the material in a ring grinder (or equivalent homogenizing device) 3 times for 2 min each, or as determined by trials (see Critical Parameters), with 2 to 3 ml of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C, to rupture any remaining unbroken cells. Monitor the extent of cell breakage using Ponceau 2R (see Basic Protocol 2, step 7).

4. Wash the preparation by first pouring the homogenate onto 11-μm nylon mesh in a Buchner funnel placed over a sturdy flask and then adding two 50-ml applications of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C. Add further HEPES/DTT buffer (25- to 50-ml aliquots) until filtrate runs clear. Monitor removal of protein with Ponceau 2R. Combine the filtrate with that from step 2 to give the water-soluble fraction. Dialyze against distilled water (e.g., 5 liters) for 3 days at 4°C, with three changes of water per day (see Support Protocol).

   Keep volumes of buffer as small as possible to minimize losses of water-soluble polysaccharides from the cell walls. Record total buffer used.

5. Gently scrape the isolated cell walls from the mesh and place in a clean vial. Record total wet weight and store at 4°C for fractionation (see Basic Protocol 4).

   Fractionation should be started within 12 to 15 hr of isolation as described in Basic Protocol 4.

   For analyses of whole cell walls remove a small aliquot, record weight, freeze dry, and weigh.

   To estimate the recovery of cell walls as dry weight, remove a small aliquot, record weight, freeze dry, and then further dry to a constant weight over desiccant.

6. Freeze dry, weigh, and analyze the resulting aqueous samples as previously described (see Basic Protocol 2, steps 14 to 16).

BASIC PROTOCOL 3

ISOLATION OF CELL WALLS FROM PLANT TISSUES THAT CONTAIN STARCH USING PHENOL-HEPES

Basic Protocol 2 was a procedure for isolating cell walls from tissues that contain very little or no starch. Many plant foods contain large amounts of starch and so consideration must be given to methods for isolating walls from such tissues. In this protocol, extraction with phenol-HEPES is followed by treatments with dimethyl sulfoxide (DMSO) and α-amylase to remove the starch.

Materials

Plant material, fresh and free from bruises and pathogens
50 mM HEPES buffer, pH 6.7 (see recipe)
Ponceau 2R (see recipe)
Iodine in potassium iodide (see recipe)
90% (v/v) dimethyl sulfoxide (DMSO)
20 mM HEPES buffer, pH 6.9/2 mM calcium chloride (see recipe)
α-amylase, porcine pancreatic type 1A (Sigma; optional)

Rotor and 250-ml HDPE centrifuge bottles
25-ml pipet
Dialysis tubing (see Support Protocol)
11-μm nylon mesh (Nybolt)
Buchner funnels
Glass-fiber filters

CAUTION: Phenol is corrosive. Gloves and safety goggles should be worn when handling. See *APPENDIX 2B* for more information.

CAUTION: DMSO is a hazardous organic chemical. Steps involving its use should be performed in a fume hood. See *APPENDIX 2B* for more information.

1. Homogenize sample as described above (see Basic Protocol 2, steps 1 to 7).
2. Transfer the mixture to a 250-ml screw-capped HDPE centrifuge bottle, using 50 mM HEPES buffer, pH 6.7 for washing, and centrifuge 20 min at 8600 × *g*, at room temperature.

   The authors currently use a Sorvall RC-28S superspeed centrifuge and the appropriate rotor; however, experimenters should use whatever is available. The most important thing is to achieve separation of the liquid and solid phases.

   If the separation of solid and liquid is incomplete centrifuge at a higher speed.

3. Remove the supernatant with a 25-ml pipet. Dialyze the phenol-HEPES-soluble fraction against running tap water for 2 days, followed by dialysis against three changes of distilled water (e.g., 5 liters), 8 hr each (see Support Protocol).

4. Resuspend the pellet in 50 ml of 50 mM HEPES buffer, pH 6.7, and centrifuge as before (step 2). Remove the supernatant and retain at 4°C. Wash the pellet two times more in 50 ml of 50 mM HEPES buffer, pH 6.7. Centrifuge, and retain the supernatant. Combine the supernatants as the water-soluble fraction and dialyze against distilled water as above.

5. Check the extent of cell wall breakage in the pellet by placing drops of the homogenate on glass slides and then adding a drop of Ponceau 2R (see Basic Protocol 2, step 7) and iodine in potassium iodide solution (see Basic Protocol 1, step 4), respectively. View with a compound light microscope.

   It is crucial that all cells break open so that all the starch granules and other cell contents are released. If cells are not broken open, then either homogenize further using the Ultra-Turrax or ring grinder.

6. Wash the cell walls with 20 ml of 90% (v/v) dimethyl sulfoxide (DMSO), then centrifuge at low speed (e.g., $1000 \times g$) 10 min at room temperature to pellet the cell walls. Decant the supernatant and retain. To gelatinize the starch, resuspend the pellet in 200 ml of 90% DMSO and incubate at room temperature with stirring for 6 hr. Centrifuge as before. Decant and retain the supernatant.

7. Resuspend the pellet in 200 ml of 90% DMSO and incubate at room temperature with stirring for 16 hr. Centrifuge as before. Decant and retain the supernatant.

8. Resuspend the pellet in 200 ml of 90% DMSO and incubate at room temperature with stirring for 24 hr.

   Monitor the gelatinization microscopically. Gelatinized granules swell and lose the blue-black staining characteristics with iodine in potassium iodide.

9. Centrifuge at low speed (e.g., $1000 \times g$) 10 min at room temperature to recover the cell walls. Resuspend the pellet in 50 ml of 20 mM HEPES buffer, pH 6.7. Wash twice more in buffer. Combine with all the DMSO supernatants and dialyze the supernatants.

   The combined supernatants are the DMSO-soluble fraction.

   IMPORTANT NOTE: *Further removal of starch may be necessary using α-amylase treatment (steps 10 and 11)*

10. *Optional:* Resuspend the pellet in 50 ml of 20 mM HEPES buffer, pH 6.9/2 mM calcium chloride. Incubate with 200 U porcine pancreatic α-amylase at 40°C for 1 hr with stirring. Filter on 11-μm mesh in a Buchner funnel. Wash twice with 50 ml HEPES/calcium chloride buffer and combine the filtrates. Dialyze against distilled water as above (step 3) and freeze dry and weigh as described in Basic Protocol 2, steps 14 to 16.

    The combined fractions are the amylase fraction.

    This step is performed only if starch is still present among the cell walls. Likewise, step 12 is also performed only if starch is still present. Starch is most likely to be present only in the phenol-HEPES and water soluble fractions since these have not been treated with DMSO; however, in this protocol, most of the starch is likely to have been centrifuged with the cell walls, and will therefore by gelatinized by the DMSO and subsequent α-amylase treatments.

11. Place the isolated cell walls in a clean vial. Record total wet weight and store at 4°C for fractionation (see Basic Protocol 4).

    Fractionation should be started within 12 to 15 hr of isolation.

For analyses of whole cell walls remove a small aliquot, record weight, freeze dry, and weigh.

To estimate the recovery of cell walls as dry weight, remove a small aliquot, record weight, freeze dry, and then further dry to a constant weight over desiccant.

12. *Optional:* Remove starch from the phenol-HEPES, water-soluble, or DMSO fractions. Centrifuge the dialyzed fractions at high speed to pellet the starch granules. Decant the supernatant. Filter supernatant through glass-fiber filter. Check filtrate with iodine in potassium iodide solution. If necessary, repeat process at higher speed of centrifugation.

13. Freeze dry and weigh the resulting aqueous samples (see Basic Protocol 2, steps 14 to 16).

Analyze the cell walls and fractions as described in UNITS E3.2, E3.3, & E3.4.

ALTERNATE PROTOCOL 2

ISOLATION OF CELL WALLS FROM TISSUES THAT CONTAIN LARGE AMOUNTS OF STARCH USING HEPES BUFFER

In this protocol additional steps are taken to eliminate starch from the isolated cell wall material to minimize erroneous estimations of glucose in later analytical procedures. In some plant material only small numbers of very tiny starch granules may be found after staining with iodine in potassium iodide (see Basic Protocol 1) and these may be deemed too few to warrant the drastic action necessary to remove the large amounts of starch found in cells of potatoes for example. If the starch granules are small in size (e.g., <10 μm), then they are likely pass though the 11-μm nylon mesh and be lost from the cell wall material; however, to remove large amounts of starch, additional treatments with dimethyl sulfoxide (DMSO) and/or α-amylase are required.

Additional Materials *(also see Basic Protocols 1 and 2)*

Plant material, fresh and free from bruises and pathogens
20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C (see recipe)
α-amylase, porcine pancreatic type 1A (Sigma)
20 mM HEPES/20 mM $CaCl_2$ (see recipe)

350-, 100-, and 54-μm Nylon mesh (Nybolt)
Ring grinder with a zirconia head (e.g., Bench Top Ring Mill; Rocklabs)
Dialysis tubing (see Support Protocol 1)

CAUTION: DMSO is a hazardous organic chemical. Steps involving its use should be performed in a fume hood. See *APPENDIX 2B* for more information.

1. Homogenize the plant tissue as described previously (see Basic Protocol 2, steps 1 to 7), substituting 20 mM HEPES buffer, pH 6.7/10 mM DTT for phenol-HEPES extraction mixture (step 4), and keeping the slurry at 4°C (on ice; step 6).

2. To remove the majority of the starch granules, pour the homogenate onto 350-μm nylon mesh in the Buchner funnel placed over a sturdy flask. Wash ten times, in 50-ml applications, with 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C. Collect the filtrate and keep at 4°C. Retain the cell walls on the mesh and keep on ice.

3. Pour the filtrate through meshes of successively smaller pore size (e.g., 100, 54, and 11 μm) to recover the smaller fragments of walls.

If the starch granules are retained by the 11-μm mesh as determined by microscopy (see Basic Protocol 1) this final filtration step should be omitted. Combine the cell walls retained on the meshes and keep on ice. Collect the filtrate and keep at 4°C.

4. Monitor the extent of cell breakage using Ponceau 2R (see Basic Protocol 2, step 7) and starch using iodine in potassium iodide (see Basic Protocol 1, step 4). If the cell wall material retained by the 350-µm nylon mesh contains unbroken cells, grind in batches of 4 to 6 g in a ring grinder (or equivalent) three times for 2 min each (as determined by trials) with 2 to 3 ml of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C to rupture the remaining unbroken cells.

5. Wash the cell walls by first pouring the homogenate onto an 11-µm (or larger if appropriate) nylon mesh in a Buchner funnel placed over a sturdy flask and then adding four 50-ml applications of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C. Add further buffer in 25- to 50-ml aliquots until filtrate runs clear. Monitor removal of protein with Ponceau 2R and starch with iodine in potassium iodide. Combine the filtrate with the filtrates from steps 2 and 3.

   Keep volumes of buffer as small as possible to minimize losses of water soluble polysaccharides from the cell walls. Record total buffer used.

   This is the water-soluble fraction.

6. Combine all the cell wall residues from steps 3 and 5.

7. Wash cell wall residues with DMSO as described for cell walls (see Basic Protocol 3, steps 6 to 9).

   The combined supernatants are the DMSO-soluble fraction.

8. *Optional:* Resuspend the pellet in 50 ml 20 mM HEPES buffer, pH 6.9/2 mM $CaCl_2$. Incubate with 200 U porcine pancreatic α-amylase at 40°C for 1 hr with stirring. Filter on 11-µm mesh in a Buchner funnel. Wash twice with 50 ml HEPES/$CaCl_2$ buffer, combine the filtrates.

   The combined filtrates are the amylase fraction.

9. Place the isolated cell walls in a clean vial. Record total wet weight and store at 4°C for fractionation (see Basic Protocol 4).

   Fractionation should be started within 12 to 15 hr of isolation.

   For analyses of whole cell walls remove a small aliquot, record weight, freeze dry and weigh.

   To estimate the recovery of cell walls as dry weight, remove a small aliquot, record weight, freeze dry, and then further dry to a constant weight over desiccant.

10. Remove starch from filtrates. Centrifuge the filtrates at high speed to pellet the starch granules. Decant the supernatant. Filter supernatant through glass fiber filter. Check filtrate with iodine in potassium iodide solution. If necessary, repeat the process at higher centrifugation speed.

11. Dialyze the water-soluble, amylase, and DMSO fractions against distilled water (e.g., 5 liters) for 3 days at 4°C, with three changes of water per day (see Support Protocol).

12. Freeze dry and weigh the fractions as described (see Basic Protocol 2, steps 14 to 16).

    Analyze whole cell walls and fractions as described in UNITS E3.2, E3.3, & E3.4.

ALTERNATE PROTOCOL 3

ISOLATION OF CELL WALLS FROM TISSUES THAT CONTAIN SMALL AMOUNTS OF STARCH USING HEPES BUFFER

A protocol describing the removal of large amounts of starch from plant material, such as maize, squash and taro, has already been described (see Alternate Protocol 2); however, other plant material may contain much smaller amounts of starch and thus less complex procedures can be used. For example, treatment with α-amylase alone may be sufficient; however, as native starch is crystalline, it must be gelatinized before α-amylase is added. This usually involves heating the material for a short time in buffer and then cooling. Unfortunately, heating in buffer is likely to solubilize some wall polysaccharides. Heating may be avoided by using 90% DMSO (see Basic Protocol 3, step 6).

Additional Materials *(also see Basic Protocols 2 and 3)*

Plant material, fresh and free from bruises and pathogens
20 mM HEPES buffer, pH 6.7, with and without 10 mM DTT (see recipes)
20 mM HEPES buffer (see recipe)/2 mM $CaCl_2$
α-amylase, porcine pancreatic type 1A (Sigma)
20 mM HEPES/20 mM $CaCl_2$ (see recipe)

54-μm nylon mesh (Nybolt)
Ring grinder with a zirconia head (e.g., Bench Top Ring Mill; Rocklabs)
Dialysis tubing (see Support Protocol)

CAUTION: DMSO is a hazardous organic chemical. Steps involving its use should be performed in a fume hood. See *APPENDIX 2B* for more information.

1. Homogenize plant tissue as described (see Basic Protocol 2, steps 1 to 7), substituting 20 mM HEPES buffer/10 mM DTT for phenol-HEPES extraction mixture in step 4, and keeping the slurry at 4°C (on ice; step 6).

2. To remove the majority of the starch granules, pour the homogenate onto 54-μm nylon mesh in a Buchner funnel placed over a sturdy flask, then wash with ten times with 50-ml applications of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C. Collect the filtrate and keep at 4°C.

3. Monitor the extent of cell breakage using Ponceau 2R (see Basic Protocol 2, step 7) and starch using iodine in potassium iodide (see Basic Protocol 1, step 4). If the solid material retained by the 54-μm nylon mesh contains unbroken cells, grind (in batches of 4 to 6 g) in a ring grinder (or equivalent) 3 times for 2 min each (as determined by trials) with 2 to 3 ml of 20 mM HEPES buffer, pH 6.7/10 mM DTT, 4°C, to rupture the remaining unbroken cells.

4. Wash the preparation by first pouring the homogenate onto 11-μm nylon mesh in a Buchner funnel placed over a sturdy flask, then adding four successive 50-ml applications of 20 mM HEPES buffer, pH 6.7 (excluding DTT), 4°C. Add further buffer in 25- to 50-ml aliquots until the filtrate runs clear. Monitor removal of protein with Ponceau 2R (see Basic Protocol 2, step 7) and starch (see Basic Protocol 1, step 4) with iodine in potassium iodide. Combine the filtrate with the filtrate from step 2.

   Keep volumes of buffer as small as possible to minimize losses of water soluble polysaccharides from the cell walls. Record total buffer used.

   This is the water-soluble fraction.

5. Combine all cell wall residues from steps 2, 4, and 5.

6. Determine the gelatinization temperature of the starch in the cell wall residue by resuspending starch in 10 ml of 20 mM HEPES buffer, pH 6.9 containing 2 mM $CaCl_2$

(excluding DTT). Incubate 10 min at 50°C. Examine with iodine in potassium iodide (see Basic Protocol 1). Repeat incubations at increasing temperatures (e.g., 5°C increments) until gelatinization occurs.

This should be done at the same time as the trials.

Gelatinized starch appears as a swollen globular mass that does not stain black.

7. Resuspend the cell wall residue in 50 ml of 20 mM HEPES buffer, pH 6.9/2 mM $CaCl_2$ (excluding DTT). Heat at the gelatinization temperature for 10 min or until all the starch has gelatinized. Cool to 40°C, add 200 U α-amylase, and stir for 1 hr at 40°C.

   It is important after gelatinization not to let the temperature fall to room temperature or below as the starch may retrograde, making it even more difficult to remove.

8. Filter the cell walls on 11-µm nylon mesh. Wash twice with 20 ml of 20 mM HEPES buffer, combining the α-amylase treatment filtrates.

   The combined filtrates are the amylase fraction.

9. Place the isolated cell wall material in a clean vial. Record total wet weight and store at 4°C for chemical fractionation (see Basic Protocol 4).

   Chemical fractionation should be started within 12 to 15 hr of isolation.

   For analyses of whole cell walls remove a small aliquot, record weight, freeze dry and weigh.

   To estimate the recovery of cell walls as dry weight, remove a small aliquot, record weight, freeze dry and then further dry to a constant weight over desiccant.

10. Remove starch from filtrates. Centrifuge the filtrates at high speed to pellet the starch granules. Decant the supernatant. Filter supernatant through a glass fiber filter. Check filtrate with iodine in potassium iodide solution. If necessary, repeat the process at higher centrifugation speed.

11. Dialyze the water-soluble and amylase fractions against distilled water (e.g., 5 liters) for 3 days at 4°C, with three changes of water per day (see Support Protocol).

12. Freeze dry, weigh, and analyze fractions as described (see Basic Protocol 2, steps 14 to 16).

    Analyze the cell walls and fractions as described in UNITS E3.2, E3.3, & E3.4.

FRACTIONATION OF PLANT CELL WALL POLYSACCHARIDES

BASIC PROTOCOL 4

The polysaccharides in cell walls can be investigated by successively treating the walls with solutions of chemicals to extract the constituent polysaccharides. These fractions can then be analyzed. The walls are treated first with solutions of chelating agents, such as *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) to remove pectic polysaccharides, followed by solutions of increasing alkaline strengths, such as 50 mM sodium carbonate (Na_2CO_3) and 1 M and 4 M KOH, which are used to extract the hemicelluloses. The residue remaining after such treatments is rich in cellulose.

Materials

Isolated cell walls (using any of the above protocols)
50 mM CDTA in 50 mM potassium acetate buffer, pH 6.5 (see recipe)
Toluene
50 mM Na_2CO_3/20 mM $NaBH_4$ (see recipe)
1 and 4 M KOH/20 mM $NaBH_4$ (see recipe)
Nitrogen gas
0.1 M ammonium acetate buffer, pH 5.2 (see recipe)
Desiccant (e.g., silica gel)

Dialysis tubing (see Support Protocol)
Freeze dryer

IMPORTANT NOTE: In plant cell walls that contain low levels of pectic polysaccharides (e.g., cereal grains) the CDTA steps are omitted, in which case start at step 5.

1. Take the isolated cell walls from 50 g fresh weight of plant material (prepared in duplicate and preferably in triplicate) and wash with 20 ml of 50 mM CDTA in 50 mM potassium acetate buffer, pH 6.5. Centrifuge at 1000 × *g* for 5 min at room temperature. Decant the supernatant and retain at 4°C after adding 1 drop of toluene.
2. Resuspend the pellet in 100 ml of 50 mM CDTA in 50 mM potassium acetate buffer, pH 6.5. Stir at room temperature for 6 hr. Centrifuge at 1000 × *g* for 10 min at room temperature. Decant the supernatant and combine with previous supernatant. Repeat and combine all three supernatants.

   The combined supernatants are the CDTA-soluble fraction.
3. Wash the pellet in 20 ml 50 mM Na_2CO_3/20 mM Na_2BH_4. Centrifuge at 1000 × *g* for 5 min at room temperature. Decant the supernatant and retain at 4°C after adding 1 drop toluene.

   $NaBH_4$ is added to alkaline solutions to reduce the aldehyde (or keto) group at the reducing end of the polysaccharides to an alcohol and prevent base peeling of polysaccharides (see Critical Parameters).
4. Resuspend the pellet in 100 ml of 50 mM Na_2CO_3 sodium carbonate/20 mM $NaBH_4$. Stir at 4°C for 16 hr and then at room temperature for 2 hr. Centrifuge 1000 × *g* for 10 min at room temperature. Decant the supernatant and retain.
5. Resuspend the pellet in 100 ml of 50 mM Na_2CO_3/20 mM $NaBH_4$. Stir at room temperature for 2 hr. Centrifuge at 1000 × *g* for 10 min at room temperature. Decant the supernatant. Combine the supernatants.

   The combined supernatants are the sodium carbonate-soluble fraction.
6. Wash the pellet in 20 ml of 1 M KOH/20 mM $NaBH_4$. Centrifuge at 1000 × *g* for 10 min at room temperature. Retain the supernatant at 4°C after adding 1 drop of toluene.
7. Resuspend the pellet in 100 ml of 1 M KOH/20 mM $NaBH_4$. Flush the suspension and tube headspace with nitrogen gas. Cap and stir at room temperature for 4 hr. Centrifuge at 1000 × *g* for 10 min at room temperature. Decant the supernatant and retain. Repeat once. Combine the supernatants.

   The combined supernatants are the 1 M KOH-soluble fraction.
8. Wash the pellet with 20 ml of 4 M KOH/20 mM $NaBH_4$. Centrifuge at 1000 × *g* for 10 min at room temperature. Retain the supernatant at 4°C after adding 1 drop toluene.
9. Resuspend the pellet in 100 ml of 4 M KOH/20 mM $NaBH_4$. Flush the suspension and tube headspace with nitrogen gas, cap, and stir at room temperature for 4 hr. Centrifuge at 1000 × *g* for 10 min at room temperature. Decant the supernatant and retain. Repeat once. Combine the supernatants with that of step 10.

   The combined supernatants are the 4 M KOH–soluble fraction.
10. Wash the pellet by resuspending in 100 ml water and stirring at room temperature for 1 hr. Centrifuge at 1000 × *g* for 10 min at room temperature. Decant the supernatant and repeat the wash process. Combine the supernatants.

    The combined supernatants are the residue wash.
11. Resuspend the pellet in 50 ml water.

    This is the final residue.

12. Dialyze the CDTA fraction against 5 liters 0.1 M ammonium acetate buffer, pH 5.2 for 1 day, with three changes of buffer per day at 4°C, followed by dialysis against 5 liters of water for 3 days, with three changes of water per day at 4°C (see Support Protocol).

    See Mort et al. (1991) for more information.

13. Adjust the pH of the sodium carbonate and KOH fractions to pH 6.5 using 1 M acetic acid and concentrated HCl, respectively. Do the same for the residue wash and final residue with 1 M HCl.

14. Dialyze these fractions against 5 liters water for 3 days with three changes of water per day, at 4°C.

    An indication of the amount of acid needed can be obtained from a trial titration of the respective reagent without sample.

    Before pouring in the extract it is useful to fill the tubing with distilled water to check for leaks then empty out the water. It may be easier to use a funnel to assist, but be careful not to have any sharp edges that may puncture the tubing. Perform the whole operation over a large beaker so that if a leak develops the dialysate will be caught.

15. Freeze dry the samples as described (see Basic Protocol 2, steps 14 to 16).

16. After the samples have been freeze dried, record weights. Dry a portion over desiccant and record weights (i.e., use the freeze dried material without further drying for analyses).

PREPARATION AND USE OF DIALYSIS TUBING

SUPPORT PROTOCOL

Dialysis is a simple clean-up or purification process to remove unwanted low-molecular-weight compounds from solutions. The dialysis membrane consists of regenerated cellulose prepared from cotton linters by the viscose process. In addition to water, the membrane also contains glycerol and small amounts of sulfur compounds, which are removed during preparation of the tubing prior to use.

Materials

0.5 M sodium carbonate (Na_2CO_3)
1 mM EDTA

12,000 to 14,000 MWCO dialysis tubing, Visking size 6, 37/32-mm diameter (e.g., Medicell International)
Large beaker or clean plastic bucket
String with a label attached
Aluminum foil

NOTE: Dialysis tubing with a lower MWCO can be used, but is more expensive. The authors currently use tubing from Medicell International, because it is competitively priced and free from defects. Other brands have been used, including Servapor (Invitrogen), but it is suggested that the experimenter inquire locally as the tubing is widely available.

Prepare dialysis tubing

1. Estimate the amount of dialysis tubing required for dialysis, allowing at least one-third of the volume for movement of water into the tubing.

2. Prepare the dialysis tubing by boiling for 10 min in 0.5 M Na_2CO_3. Rinse well with distilled water and then boil for 10 min in 1 mM EDTA. If necessary, store 2 to 3 hr at 4°C in 1 mM EDTA.

Perform dialysis

3. Rinse the prepared tubing well in distilled water before use, ensuring water flushes through the tubing as well as over the outside.

4. Tie two separate knots close together at one end of the tubing. Carefully pour the extract to be dialyzed into the tube. Squeeze out the excess air in the tube above the sample and tie off the tubing again with two separate knots.

   Before pouring in the extract it is useful to fill the tubing with distilled water to check for leaks, and then empty out the water. It may be easier to use a funnel to assist, but be careful not to puncture the tubing with any sharp edges. Perform the whole operation over a large beaker so that if a leak does develop, the sample will be caught.

   Antimicrobial agents such as sodium azide or toluene (1 to 2 drops) are sometimes added into the dialysis tubing with the solution.

5. Attach a piece of string with a label attached (similar to a luggage label) to one of the knotted ends of the tubing. Place the tubing into a bucket of distilled water, 4°C draping the label over the side. Attach label to bucket with adhesive tape. Loosely cover the bucket with a sheet of aluminum foil (or similar) to prevent unwanted material falling in.

6. Dialyze against water at 4°C for 3 days with three changes of water per day.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ammonium acetate buffer, 0.1 M, pH 5.2

Dissolve 7.708 g of ammonium acetate (mol. wt. 77.08) in 950 ml of water. Adjust to pH 6.5 with glacial acetic acid. Add water to 1 liter. Store up to 24 hr at 4°C

CDTA, 50 mM, in 50 mM potassium acetate buffer, pH 6.5

Dissolve 4.55 g *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA; mol. wt. 364.35) in 200 ml water, adding saturated KOH dropwise to assist dissolution, and to bring the pH close to 6.0. Add 1.23 g potassium acetate (mol. wt. 98.14). Adjust the pH to 6.5 with further KOH and the final volume to 250 ml with water. Store up to 24 hr at 4°C.

HEPES buffer, 0.5 M, 50 mM, 20 mM

Combine 2.98 g HEPES (mol. wt. 238.30) and ~200 ml water in a beaker. Stir and carefully adjust to pH 6.7 or 6.9 using saturated KOH to get to the pH range, and then 1 M KOH to adjust to the exact pH. Adjust the final volume to 250 ml with water. Dilute with water to desired concentration. Store up to 24 hr at 4°C.

Also see Table A.2A.2 in APPENDIX 2A for more information on HEPES.

HEPES buffer, 20 mM/$CaCl_2$, 20 mM

Combine 0.476 g HEPES (mol. wt. 238.30) and ~70 ml water in a beaker. Stir and carefully adjust to pH 6.7 using saturated KOH to get to the pH range, and then 1 M KOH to adjust to the exact pH. Add 0.022 g $CaCl_2$ (mol. wt. 110.99). Stir until dissolved, then adjust the final volume to 100 ml with water. Store up to 24 hr at 4°C.

Also see Table A.2A.2 in APPENDIX 2A for more information on HEPES.

HEPES buffer, 20 mM/DTT, 10 mM

Combine 1.19 g HEPES (mol. wt. 238.30) and ~200 ml water in a beaker. Stir and carefully adjust to pH 6.7 using saturated KOH to get to the pH range, and then

continued

1 M KOH to adjust to the exact pH. Add 0.386 g DTT (mol. wt. 154.24), continue to stir adjusting the final volume to 250 ml with water.

The HEPES buffer can be made up 24 hr in advance and stored at 4°C but the dithiothreitol should be added on the day of cell wall isolation. Do not store after DTT is added.

Also see Table A.2A.2 in APPENDIX 2A for more information on HEPES.

Iodine in potassium iodide

Mix 0.2 g iodine and 2 g potassium iodide in water to give a final volume of 100 ml. The solution is stable for several months at room temperature.

For more information see Jensen (1962).

KOH, 1 and 4 M/20 mM $NaBH_4$

Dissolve 14 g (1 M) or 56.1 g (4 M) KOH (mol. wt. 56.11) in 200 ml of water. Add 0.19 g $NaBH_4$ (mol. wt. 37.83). Adjust the final volume to 250 ml. Prepare fresh.

The KOH is prepared on the day of use and the $NaBH_4$ should be added just prior to use.

Phenol-HEPES extraction mixture

Prepare by mixing 40 ml 0.5 M HEPES buffer (see recipe) with 160 g phenol.

CAUTION: *Phenol is corrosive. Gloves and safety goggles should be worn when handling. See APPENDIX 2B for more information.*

IMPORTANT NOTE: *Phenol should be colorless. Do not use orange or colored phenol. The phenol-HEPES extraction mixture should be prepared no more than 24 hr in advance and preferably on the day of use.*

Phloroglucinol

Dissolve 2 g 1,3,5-trihydroxybenzene (phloroglucinol) in 100 ml 95% ethanol. Store in the dark for ~1 month at room temperature.

Phloroglucinol·HCl solution

Mix 1 ml of a solution of phloroglucinol (see recipe) with 2 ml concentrated HCl. Prepare fresh daily and keep in the dark.

CAUTION: *HCl is very corrosive. Take special care to prevent contact with skin and microscope objectives. Also take care not to contact microscope objectives with nail varnish. See APPENDIX 2B for more information.*

Ponceau 2R

Dissolve 0.2 g Ponceau 2R in 100 ml of water, adding 2 drops of 18 M H_2SO_4. Store up to 6 months at 4°C.

Ponceau 2R stains proteins red and thus unbroken cells can be detected from the staining of their cytoplasmic contents (Harris, 1983).

Na_2CO_3, 50 mM/20 mM $NaBH_4$

Dissolve 1.32 g of Na_2CO_3 (mol. wt. 105.99) in 200 ml of water in a beaker. Just prior to use add 0.19 g $NaBH_4$ (mol. wt. 37.83) and adjust the final volume to 100 ml with water. Prepare fresh.

The Na_2CO_3 can be prepared on the day of use and the $NaBH_4$ added just prior to use.

Toluidine blue O, 0.05% (w/v)

Dissolve 0.25 g benzoic acid and 0.29 g sodium benzoate in 200 ml water. Add 0.1 g toluidine blue O and stir (Feder and O'Brien, 1968). Filter through an glass microfiber filter. Store up to several months at room temperature. Discard if a precipitate forms.

COMMENTARY

Background Information

Cell wall isolation

The aim of the cell wall isolation steps (see Basic Protocol 2) is to break open the plant cells and wash away the cell contents, leaving the cell walls unaltered; however, because of the complex nature of plant cell walls, their isolation involves some compromises. Older methods of isolating cell walls, particularly from dicots, were concerned with inactivating enzymes in the cell walls so that they would not alter the cell wall polysaccharides during the isolation procedure. The plant tissue was homogenized in 70% or 80% ethanol, but unless the ethanol was boiling there was no expectation that the enzymes would be fully inactivated (Rose et al., 1998), as even boiling in 80% ethanol may not be enough to completely inactivate polygalacturonase (Huber, 1991). Moreover, ethanol treatment can result in contamination by proteins, starch, and RNA (Fry, 1988). In addition, any procedure that dehydrates the cell walls will change the physical nature of the cell walls (Sasaki and Nagahashi, 1989; Selvendran and Ryden, 1990) and alter the solubility of the component polysaccharides in the subsequent sequential fractionation of the wall.

A 2:1:1 (v/v/v) mixture of phenol:acetic acid:water (PAW) has been commonly used to inactivate enzymes during cell wall isolation (Redgwell et al., 1988; Selvendran and Ryden, 1990). Phenol solubilizes some of the wall structural proteins as well as enzymes. The PAW procedure may well inactivate enzymes, but it may also alter the physical nature of the cell wall polysaccharides. For instance, PAW alters pectin solubility by removing calcium ions from the walls (Huber, 1991). Another disadvantage of the PAW method is that the acetic acid is not effective at buffering the phenol to maintain the pH. Moreover, the pH is altered when the temperature is lowered during extraction. This has led to adopting an 80:20 (w/v) HEPES buffered-phenol mixture (e.g., Koh et al., 1997; Smith et al., 1998). The HEPES buffer has the advantage that it resists pH change with lowering temperature and it does not complex calcium ions, which cross-link pectic polygalacturonic acid molecules, and so hold them in the cell walls. The alternative approach to cell wall isolation is to use a very mild extraction procedure, avoiding dehydration and strong chemicals, with the aim of obtaining cell walls that are not physically or chemically altered during the isolation. By necessity, this means the cell walls must be kept hydrated at all times. For this, the authors use HEPES buffer without phenol (Smith and Harris, 1995; Ratnayake et al., 1999). This procedure, like all others, is a compromise in that near-zero temperature and swift handling are relied on to inhibit the activities of cell wall enzymes. With mild aqueous extraction of apples, endogenous pectic methylesterase was active to some extent (L.D. Melton and M.B. O'Neill, unpub. observ.) so this method should not be used if the degree of methylation of pectins is of interest.

Starch requires another compromise. If starch is present in large amounts it must be actively removed or else there may be more starch than cell walls, and the carbohydrate analyses will be meaningless. On the other hand, the more extensive the procedure for removing starch, the greater the loss of water-soluble polysaccharides [e.g., pectins or some (1→3,1→4)-β-D-glucans]. If the starch level is ≤1% of the fresh tissue by weight, its presence may be ignored in order to obtain a more complete cell wall isolate. Three procedures for removing starch have been given. For lower levels (i.e., 2% to 5%) the procedure is relatively straightforward; however, for higher starch content numerous steps may be necessary (Ratnayake et al., 1999).

When dealing with starch there are two essential things to remember.

1. Starch cannot be fully removed unless the cell walls are broken open.
2. α-amylase cannot completely degrade starch unless the starch has been gelatinized. Gelatinization can be accomplished by heating to the gelatinization temperature of the particular starch (usually between 80° and 90°C) or by using 90% DMSO. Use a microscope to ensure that gelatinization is complete. Porcine pancreatic α-amylase is used (Ferguson et al., 1992) as it is unlikely to be contaminated with enzymes that might degrade cell wall polysaccharides.

Fractionation of cell wall polysaccharides

Selvendran and his coworkers (Selvendran et al., 1985; Redgwell and Selvendran, 1986) had a major influence on developing the sequential extraction of polysaccharides from primary cell walls. The procedure starts with extracting the water-soluble pectic polysaccha-

rides, then a chelating agent (CDTA) is used to extract the Ca^{2+} ions which cross-link polygalacturonic acids, allowing them to solubilize. Alkaline solutions of increasing concentrations are then used to sequentially extract the hemicelluloses [e.g., xyloglucans, arabinoxylans, and (1→3,1→4)-β-D-glucans] from the insoluble residues. Alkalis cleave hydrogen bonds and ester linkages. The final insoluble residue contains cellulose. Older fractionation protocols tended to use a wide range of extractants, including DMSO, urea, and guanidinium thiocyanate, while the current approach is to simplify the procedure and rely on increasing alkali concentration to achieve the desired separation (Carpita, 1984; Smith and Harris, 1995; Rose et al., 1998; Ratnayake et al., 1999).

The fractionation procedure results in some fractions containing more than one type of polysaccharide. Pectic polysaccharides with high galacturonic acid content will be found in the CDTA fraction, while pectic polysaccharides with higher neutral sugar levels will be extracted by the sodium carbonate and KOH solutions. Moreover, some pectic polysaccharide is invariably found in the cellulose residue fraction as is some hemicellulose. Hemicelluloses are found in all alkaline extracts, including the sodium carbonate. After 4 M KOH treatment of dicot walls, if the residue is washed with water, a significant amount of xyloglucan can be solubilized. It appears that the 4 M KOH treatments loosens the remaining cell wall structure but the xyloglucan is more soluble in water than 4 M KOH. If the mixture of polysaccharides in a fraction has to be separated, ion exchange or gel filtration chromatography can be used.

Finally, the experimenter should be aware that the cell walls from each plant and each plant tissue are different and require their own cell wall isolation and fractionation protocols. Hence for any given plant material, modifications of the basic procedures described in this unit will probably be required.

Critical Parameters

All cells have to be broken open to remove the cell contents. To accomplish cell lysis the authors use a combination of homogenizing and grinding steps. For recalcitrant tissue a small rock grinder (i.e., ring grinder) is used. The authors advise the experimenter to perform a trial run on their plant material to establish the number and duration of homogenization steps. These should always be the minimum required to break open the cells. Excessive homogenization will lead to the formation of cell wall fragments which may not be recovered. In addition, the speed and duration of centrifugation should be the minimum required to form a firm but not hard pellet. When phenol-HEPES is used as the homogenizing medium, the centrifugation speed is as high as needed to achieve a hard pellet so that the phenol can be removed effectively with minimum handling. In other situations, a lower speed is all that is required to pellet the cell walls, in the hope that intracellular material remains in the supernatant and only the walls are brought down.

When phenol is not included in the extraction mixture, enzyme degradation of cell walls during isolation is avoided by keeping the material initially frozen with liquid nitrogen (storage of material should be at –80°C) and subsequently not allowed to rise above 4°C during the cell wall isolation. This means all containers have to be precooled and kept cold. It is good practice to proceed to fractionation immediately after cell wall isolation.

It should be appreciated that the water-soluble polysaccharides obtained during cell wall isolation are part of the cell wall and are to be treated as such. Take care with older isolation procedures as the water-soluble fractions were often discarded.

Sodium borohydride (NaBH4) must be added to the alkaline extractions used in the cell wall fractionation or degradation of the polysaccharides will occur by base-peeling (Aspinall, 1982). In alkali, the sugar at the reducing end rearranges until it is cleaved from the polysaccharide and thus exposes a new reducing end. In this manner the polysaccharide is degraded one monosaccharide unit at a time. Base-peeling is more severe in the presence of oxygen. The alkaline extractants will hydrolyze esters and so the solubilized pectic polysaccharides will be devoid of their methyl and acetate groups.

All fractions should have their combined neutral sugars and uronic acid (and cellulose if appropriate) contents checked against the weight of the fraction. Except for the initial fractions, which contain protein and other cellular components, the combined carbohydrate analyses figures should agree approximately with the weight of the fraction.

Troubleshooting

With the phenol-HEPES wall isolation, the soluble material in the phenol-HEPES should contain relatively little carbohydrate content compared to subsequent fractions. The same is

true of the HEPES-buffer wall isolation. In the later case, the amount of HEPES buffer used should be kept to a minimum to avoid water-soluble polysaccharides being removed from the wall.

CDTA cannot be removed from the CDTA-soluble fraction by standard dialysis against water (Mort et al., 1991). Therefore, it is essential to follow the special dialysis procedure for this fraction. Mort et al. (1991) found that removal of CDTA could be greatly improved by initial dialysis against ammonium acetate buffer followed by dialysis against water. If CDTA has not been removed, the weight of the fraction will be clearly in excess of its carbohydrate content.

To avoid contamination by cellulose, do not filter through filter paper or Miracloth, instead use nylon mesh or glass fiber filters.

It is essential to analyze all fractions as a check on the validity of the chosen procedure.

Anticipated Results

Dried isolated cell walls should be white or creamy-white in color, light, and fluffy.

From 50 g fresh weight of parenchyma tissue which does not contain starch, the expected recovery is ~400 mg of cell walls, whereas, from 50 g fresh weight of parenchyma tissue containing a large amount of starch, the yield is ~300 to 350 mg. The yield of cell walls from collenchyma tissue would be much higher due to the thicker cell walls, and yields of ~6 g have been obtained from 50 g fresh weight.

Dried fractions should be white. The proportions recovered by the different chemical treatments will be different, depending on the composition of the cell walls; however, traditionally, most of the pectic polysaccharides are extracted in the water-soluble fractions (during cell wall isolation), CDTA, and sodium carbonate fractions, but a small proportion is often found in the final residue. Hemicelluloses are usually extracted by 1 M and 4 M KOH. The final residue is predominantly cellulose.

Time Considerations

Allow plenty of time to study the macroscopic and microscopic appearance of the material.

Isolation of cell walls should be completed in 1 day, and the fractionation should be started immediately after the isolated walls have been prepared and continued until completed. Realistically, fractionation may have to start the following day and the preparation stored overnight at 4°C. The whole fractionation process will take ~1 week.

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Key References

Fry, 1988. See above.

An outstanding book full of useful information on plant cell walls including their extraction fractionation and analysis. It may be out of print but is available in most good libraries.

Harris, 1983. See above.

An excellent review of the older methods of isolating cell walls. Contains numerous insightful comments.

Huber, 1991. See above.

Describes how different procedures affect the solubility of pectic polysaccharides.

Mort et al., 1991. See above.

A useful paper dealing with problems that might be encountered with pectic polysaccharides.

Selvendran, Stevens, and O'Neill, 1985. See above.

A classic. If there is a desire to gain a feel for the developments in the field of cell wall isolation and fractionation, this is a good place to begin.

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Determination of Neutral Sugars by Gas Chromatography of their Alditol Acetates

The neutral monosaccharide composition of cell walls can be determined by first hydrolysing the polysaccharides to their constituent monosaccharides with strong acid and then converting them to alditol acetates by reduction with sodium borohydride to the corresponding alditol, followed by acetylation of the hydroxyls on each alditol. The resultant alditol acetates are volatile and can be identified and quantified by gas chromatography. Thus, a gas chromatograph is essential for this procedure. The amounts of the individual monosaccharides in the cell walls can be determined and then summed to give the total neutral sugar content. While this procedure does not identify the parent polysaccharides, some inferences can be made since the types of polysaccharides in cell walls are well known.

Several procedures have been used to hydrolyze polysaccharides in cell walls and cell wall fractions. For example, the noncellulosic polysaccharides can be hydrolyzed using 1 M sulfuric acid for 2 to 3 hr at 100°C (Selvendran and Ryden, 1990). One of the simplest procedures is that of Albersheim et al. (1967) in which hydrolysis of the noncellulosic polysaccharides is achieved by incubating in 2 M trifluoroacetic acid (TFA) at 121°C for 1 hr. The advantage of the TFA procedure is that it is quick and the acid can be removed by evaporation in a gentle stream of air or nitrogen. However, neither the 1 M sulfuric acid or TFA procedures hydrolyze cellulose. Hydrolysis of cellulose can be achieved by an initial dispersion in 72% (w/w) sulfuric acid (Saeman et al., 1963; Selvendran et al., 1979; Fry, 1988; Harris et al., 1988; Selvendran and Ryden, 1990) followed by hydrolysis in 1 M sulfuric acid.

Determination of the monosaccharide composition by gas chromatography of alditol acetates is commonly used for cell wall analyses because the procedure gives a single peak for each sugar. An alternative is to prepare trimethylsilyl (TMS) derivatives. However, these are easily hydrolyzed by moisture in the atmosphere and therefore should be analyzed in the gas chromatograph immediately, whereas alditol acetates are relatively more stable and could be rerun on the gas chromatograph the following day if required.

This unit provides two protocols (see Basic Protocols 1 and 2) and an alternative procedure (see Alternate Protocol) for estimating neutral sugars. Differences among these protocols are in the method of hydrolysis. The choice of method will depend on the type of information required. Basic Protocol 1 is used when only the amounts of noncellulosic neutral sugars are required. When both the noncellulosic neutral sugars and the cellulose content are required, then Basic Protocol 2 or Alternate Protocol 1 are used.

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS BY TRIFLUOROACETIC ACID (TFA) HYDROLYSIS

BASIC PROTOCOL 1

Hydrolysis with TFA is rapid and gives yields of monosaccharides that are equal to those achieved by mineral acids (Albersheim et al., 1967). The great advantage of using TFA is that it can be readily removed by evaporation, i.e., under a gentle stream of clean nitrogen or air.

The preferred method for reduction and acetylation is that of Blakeney et al. (1983). Their innovation was to use DMSO as the solvent for sodium borohydride and to use 1-methylimidazole as the catalyst for the quantitative acetylation of alditols in the presence of borate. Methods commonly used to prepare alditol acetates prior to this had incorporated steps in which the borate was removed by repeated evaporations with methanol, which was slow and tedious.

Contributed by Laurence D. Melton and Bronwen G. Smith
Current Protocols in Food Analytical Chemistry (2001) E3.2.1-E3.2.13

Materials

Dried cell walls or cell-wall fractions (*UNIT E3.1*)
2 M trifluoroacetic acid (TFA; see recipe)
Nitrogen or argon gas
20 mg/ml allose
13-sugar standard (see recipe)
15 M (concentrated) ammonia (analytical grade)
0.5 M sodium borohydride in DMSO (freshly prepared; see recipe)
18 M acetic acid (glacial, analytical grade)
1-methylimidazole
Acetic anhydride
Dichloromethane (DCM; high quality)
Helium (zero grade; carrier gas for GC)

15-ml borosilicate glass tubes and caps with Teflon-lined insert
5-ml glass syringe
Swinney stainless steel 13-mm filter unit (Millipore)
0.22-µm polytetrafluoroethylene (PTFE) filters (Millipore) or glass fiber filters
40°C water bath
Gas chromatograph (e.g., Model HP 6890, Hewlett Packard) fitted with a flame ionization detector and a dedicated cool on-column capillary inlet
BPX-70 open tubular fused silica column (25 m long, 0.33 i.d., and 0.25-µm film thickness; SGE Chromatography Products)
Hewlett Packard ChemStation software

Hydrolyze cell walls or cell-wall fractions with TFA

1. Place 5 mg of dried cell walls or cell-wall fractions (*UNIT E3.1*) into a scrupulously clean (see Critical Parameters) borosilicate glass tube (in duplicate). Record the exact weight of cell walls or fractions.

2. Add 0.5 ml of 2 M TFA to each sample. Set up a tube containing only 0.5 ml of TFA as a control and take this through all the following steps.

   CAUTION: *TFA is a strong acid—wear goggles, gloves, and protective clothing.*

   A clean glass pipet is recommended to deliver the acid, as accidental splashes and fumes from acids can cause corrosion of expensive automatic pipets.

3. Flush tubes well with argon or nitrogen gas (to remove all traces of air) and cap tightly using a screw cap with Teflon-lined insert. Vortex to mix, taking care not to spread the solid material above the level of the liquid.

4. Incubate for 60 min at 121°C. Allow to cool.

   A heating block may be used for the incubations.

5. Add 25 µl of 20 mg/ml allose (internal standard). Vortex to mix.

6. Filter hydrolysate using glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube. After each sample has been filtered, discard the PTFE filter and wash the syringe and filter unit 6 times in Milli-Q-purified water to ensure there is no carryover from one sample to another.

   Alternatively, glass-fiber filters can be used, but they should be heated to 500°C in an oven for 1 hr to remove all traces of contaminating carbohydrates prior to use.

7. Evaporate filtrate to dryness in a gentle stream of air or nitrogen gas.

   Use new, washed Pasteur pipets (or needles) to ensure there are no traces of debris, especially from cardboard boxes in which the pipets are transported.

Reduce monosaccharides to corresponding alditols

8. Take dried hydrolysates and add 100 µl Milli-Q-purified water to each tube.
9. Set up two clean tubes as controls. Add 100 µl Milli-Q water (water control) to one tube and 100 µl of the 13-sugar standard to the other tube.

   Allow the standard to thaw and mix well before use.

10. Add 20 µl of 15 M ammonia to each tube.

    Do this step in a fume hood.

11. Add 1 ml of 0.5 M sodium borohydride in DMSO to each tube. Cap the tubes and vortex to mix.
12. Incubate for 90 min at 40°C.

    Use either a heating block or a water bath for incubation.

13. Add 100 µl of 18 M acetic acid to each tube. Vortex to mix.

    Do this step in a fume hood. The mixture should effervesce as the sodium borohydride is destroyed.

Acetylate the alditols

14. Add 200 µl of 1-methylimidazole.

    The 1-methylimidazole is stored over silica gel at 4°C. Allow it to come to room temperature before opening the bottle. Decant a small volume into a vial and use this rather than putting pipet into bottle.

15. Add 2 ml acetic anhydride to each tube and vortex to mix.
16. Incubate for 10 min at room temperature.
17. Add 5 ml Milli-Q-purified water to each tube to destroy the excess acetic anhydride. Vortex to mix.
18. Incubate for 10 min at room temperature or until cool.
19. Add 1 ml dichloromethane (DCM) to extract the alditol acetates. Vortex to mix (do not cap the tubes, as the glue holding Teflon liners may be soluble in DCM). Allow the phases to separate and transfer the lower DCM phase to a clean borosilicate glass tube using a Pasteur pipet.

    Do this by first expelling the air from the Pasteur pipet and then lowering it through the aqueous phase and into the DCM phase. Take care to ensure that very little or preferably none of the aqueous phase enters the pipet. The DCM is volatile and tends to squirt out of the pipet; hold both tubes together in one hand so that it is possible to transfer the DCM quickly without losses.

20. Add another 1 ml DCM to the original solution (aqueous phase) and repeat the extraction process.

    If necessary, centrifuge at slow speed for ~2 min to separate the phases. Remove the lower DCM phase and combine the DCM extracts.

21. Add 4 ml Milli-Q-purified water to the combined DCM extracts and vortex to mix. Remove upper aqueous phase and discard. Add 4 ml water and repeat the wash procedure an additional three times.

    If necessary, centrifuge at slow speed for ~2 min to separate the phases. After the last wash, centrifuge as above and remove the lower DCM phase to a clean glass vial (caps should be Teflon-lined).

22. Gently evaporate the DCM completely in a stream of instrument-grade air or nitrogen gas, and add 2 ml of DCM. Proceed to gas chromatography of the fully acetylated alditols.

    Take care when evaporating the DCM, as some alditol acetates are particularly volatile—see Troubleshooting.

Run gas chromatography

23. Separate and quantify alditol acetates using a gas chromatograph (e.g., Model HP 6890, Hewlett Packard), fitted with a flame ionization detector and a dedicated cool on-column capillary inlet (set on "oven track mode") on a BPX-70, open tubular fused silica column (25 m long, 0.33 i.d. and 0.25-µm film thickness).

    For an alternative column and gas chromatography system see Carrington et al. (1993).

    a. Set the initial oven temperature at 38°C and maintain it for 30 sec, increase to 170°C at 50°C/min, and then increase to 230°C at 2°C/min and hold at 230°C for 5 min.

    The detector temperature is held at 250°C. Helium (zero grade) is used as the carrier gas at a column head pressure of 40 kPa. The flow rate of the other gases are: hydrogen, 40 ml/min; air, 450 ml/min; and nitrogen, 45 ml/min. Total run time is 38 min. The results are integrated using Hewlett Packard ChemStation software.

    b. Inject 0.5 µl of samples and controls.

    Reference alditol acetates elute from the BPX-70 column in the following order: erythritol triacetate, 2-deoxyribitol tetraacetate, rhamnitol pentaacetate, fucitol pentaacetate, ribitol pentaacetate, arabinitol pentaacetate, xylitol pentaacetate, 2-deoxyglucitol hexaacetate, allitol hexaacetate, mannitol hexaacetate, galactitol hexaacetate, glucitol hexaacetate, and myo-inositol hexaacetate. Their identification can be verified by using individual standards.

24. Identify the alditol acetates by the retention times relative to the standards in the 13-sugar standard.

    The amount of each neutral monosaccharide in the samples can be calculated relative to the internal standard, allose, using response factors. The relative response of the detector for the individual alditol acetates can be calculated from the areas under the peaks for each alditol acetate, relative to the area under the peak for allitol acetate. There should be no peaks in the chromatogram of the water control and only one peak, corresponding to allitol hexaacetate from the internal standard allose, in the chromatogram of the TFA control.

BASIC PROTOCOL 2

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS AND CELLULOSE CONTENT BY TRIFLUOROACETIC ACID HYDROLYSIS FOLLOWED BY SULFURIC ACID HYDROLYSIS

In this procedure, the cell walls are first treated with TFA to determine the neutral monosaccharide composition of the noncellulosic polysaccharides. The TFA-insoluble residue is then hydrolyzed using a two-stage sulfuric acid procedure to determine cellulose content.

Materials

Dried cell walls or final residue from cell wall fractionation (see *UNIT E3.1*)
Methanol
Nitrogen gas
72% (w/w) sulfuric acid (see recipe)
Milli-Q-purified water
15 M ammonia (analytical grade)
20 mg/ml allose
13 sugar standard (erythritol, 2-deoxyribose, rhamnose, fucose, ribose, arabinose, xylose, 2-deoxyglucose, allose, mannose, galactose, glucose, *myo*-inositol)

15-ml borosilicate glass tubes and screw-caps with Teflon-lined inserts
100°C and 30°C heating block or bath
5-ml glass syringe
Swinney stainless steel 13-mm filter unit (Millipore)
0.22-µm polytetrafluoroethylene (PTFE) filters or glass fiber filters
Gas chromatograph fitted with a flame ionization detector and a dedicated cool-on-column capillary inlet

Additional reagents and equipment for hydrolysis with TFA (see Basic Protocol 1)

Hydrolyze cell walls or final residue fraction using TFA

1. Perform TFA hydrolysis (see Basic Protocol 1, steps 1 to 5).

2. Add 2 ml methanol and centrifuge at 1000 × *g*, room temperature. Remove the supernatant using a Pasteur pipet and place in a clean glass tube.

   Use new, washed Pasteur pipets to ensure there are no traces of debris, especially from cardboard boxes that the pipets are transported in.

3. Add 2 ml methanol to pellet, vortex to mix, and centrifuge as in step 2. Remove the supernatant and combine with the supernatant from step 2. Evaporate the combined supernatants to dryness in a gentle stream of air or nitrogen gas. Proceed with reduction in step 5.

4. Retain the pellet, i.e., TFA-insoluble material, and proceed with two-stage sulfuric acid hydrolysis in step 6.

Reduce TFA-soluble material

5. Reduce TFA-soluble material in the combined supernatants from step 3 (see Basic Protocol 1, steps 8 to 13), then proceed with acetylation (see Basic Protocol 1, steps 14 to 22).

Hydrolyze TFA-insoluble residue with a two-stage sulfuric acid hydrolysis

6. Add 125 µl of 72% sulfuric acid to the TFA-insoluble residue pellet from step 4 using a glass pipet. Set up a control tube containing 125 µl of 72% sulfuric acid and take this through all the following steps.

   CAUTION: *Sulfuric acid is very corrosive. Wear goggles, gloves, and protective clothing.*

7. Flush tubes well with argon or nitrogen gas to remove all traces of air and cap using a screw-cap with Teflon-lined insert. Vortex to mix (take care not to splatter the solid material above the level of the liquid).

8. Incubate for 3 hr at 30°C. Vortex to mix at frequent intervals to aid dissolution (take care not to splatter the solid material above the level of the liquid). Allow to cool.

9. Add 1.375 ml Milli-Q-purified water, then mix thoroughly and quickly. Flush with argon or nitrogen gas. Cap tightly. Incubate for 3 hr 100°C. Allow to cool.
10. Add 300 µl of 15 M ammonia to neutralize the solution. Vortex to mix.

    Do this step in a fume hood.

    IMPORTANT NOTE: *Sugars are unstable when left in alkaline solutions, so proceed with next steps quickly.*
11. Add 50 µl of 20 mg/ml allose (internal standard). Vortex to mix.
12. Filter the hydrolysate using glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube.

    After a sample has been filtered, discard the PTFE filter and wash the syringe and stainless steel filter unit six times in Milli-Q-purified water to ensure there is no carryover from one sample to another.

    Alternatively glass fiber filters can be used, but they should be heated to 500°C in an oven prior to use to remove all traces of contaminating carbohydrates.
13. Add 200 µl of the filtered hydrolysate to a scrupulously clean borosilicate glass tube (see Critical Parameters).

Prepare controls

14. Prepare a water control by adding 200 µl Milli-Q-purified water to a clean borosilicate glass. Add 20 µl of 15 M ammonia and vortex to mix.
15. Prepare a 13-sugar standard control by adding 100 µl of the 13-sugar standard to a clean borosilicate glass tube (allow the standard to thaw and mix well before use), then add 100 µl of Milli-Q-purified water (total volume 200 µl) and 20 µl of 15 M ammonia. Vortex to mix.

Reduce and acetylate sugars from TFA-insoluble material

16. Reduce the monosaccharides in the filtered hydrolysates and controls to the corresponding alditols (see Basic Protocol 1, steps 11 to 13). Acetylate the alditols (see Basic Protocol 1, steps 14 to 22).

Run gas chromatography

17. Separate and quantify alditol acetates for both the TFA-soluble (step 5) and insoluble material (step 16) using a gas chromatograph (see Basic Protocol 1, steps 23 to 24).

ALTERNATE PROTOCOL

DETERMINATION OF NONCELLULOSIC NEUTRAL SUGARS AND CELLULOSE CONTENT BY SULFURIC ACID HYDROLYSIS

This protocol is an alternative to Basic Protocol 2 and can be used to fully hydrolyze cellulose as well as other polysaccharides simultaneously. In this protocol a two-stage sulfuric acid procedure is used (Harris et al., 1988). This procedure is simpler than that described in Basic Protocol 2; however, it has the disadvantage of some noncellulosic neutral sugars being degraded during cellulose hydrolysis. Moreover, one cannot tell the source of glucose (e.g., whether it comes from cellulose or xyloglucan).

For materials see Basic Protocol 2.

Hydrolyze by two-stage sulfuric acid hydrolysis

1. Place 10 mg of dried cell walls or final residue fraction (*UNIT E3.1*) into a scrupulously clean (see Critical Parameters) borosilicate glass tube (in duplicate). Record the exact weight.

   This procedure can be done using 5 mg of cell walls or fraction and half volumes of reagents to step 6.

2. Add 250 µl of 72% sulfuric acid using a glass pipet. Set up a tube containing 250 µl of 72% sulfuric acid as a control and take this through all the following steps.

   A clean glass pipet is recommended to deliver the acid, as accidental splashes and fumes from acids can cause corrosion of expensive automatic pipets.

   CAUTION: *Sulfuric acid is very corrosive—wear goggles, gloves, and protective clothing.*

3. Flush tube well with argon or nitrogen gas to remove all traces of air and cap using a screw-cap with Teflon-lined insert. Vortex to mix (take care not to splatter the solid material above the level of the liquid).

4. Incubate for 3 hr at 30°C. Vortex to mix at frequent intervals to aid dissolution (take care not to splatter the solid material above the level of the liquid). Allow to cool.

5. Add 2.75 ml Milli-Q-purified water mix thoroughly and quickly. Flush with argon or nitrogen gas, cap tightly, and incubate for 3 hr at 100°C. Allow to cool.

6. Working in a fume hood, add 600 µl of 15 M ammonia to neutralize the solution. Vortex to mix.

   IMPORTANT NOTE: *Sugars are unstable when left in alkaline solutions so proceed quickly.*

7. Add 50 µl of 20 mg/ml allose (internal standard). Vortex to mix.

8. Filter the hydrolysate using a glass syringe fitted with a Swinney stainless steel 13-mm filter unit and a 0.22-µm PTFE filter into a clean borosilicate glass tube.

   After a sample has been filtered, discard the PTFE filter and wash the syringe and filter unit six times with Milli-Q-purified water to ensure there is no carryover from one sample to another.

   Alternatively, glass-fiber filters can be used, but they should be heated to 500°C in an oven prior to use to remove all traces of contaminating carbohydrates.

9. Add 200 µl of the filtered hydrolysate to a scrupulously clean borosilicate glass tube (see Critical Parameters).

Prepare controls

10. Prepare a water control by adding 200 µl Milli-Q-purified water to a clean borosilicate glass. Add 20 µl of 15 M ammonia and vortex to mix.

11. Prepare a 13-sugar standard control by adding 100 µl of the 13-sugar standard to a clean borosilicate glass tube (allow the standard to thaw and mix well before use), then add 100 µl Milli-Q-purified water (total volume 200 µl) and 20 µl of 15 M ammonia. Vortex to mix.

Reduce and acetylate sugars

12. Reduce monosaccharides to corresponding alditols (see Basic Protocol 1, steps 11 to 13).

13. Acetylate the alditols (see Basic Protocol 1, steps 14 to 22).

Run gas chromatography

14. Separate and quantify alditol acetates using a gas chromatograph as described in Basic Protocol 1, steps 23 to 24.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Sodium borohydride in DMSO, 0.5 M

Weigh out 0.5 g sodium borohydride into a glass bottle with a Teflon-lined lid. Add 25 ml of DMSO (use the highest quality only, if necessary dry over molecular sieves) loosely cap and place in oven for 1 hr at 100°C, with occasional gentle swirling. Remove from oven, tighten cap, cool, and store in dark (or wrap in aluminum foil). Prepare fresh on the day of use.

Take care when handling, wear gloves, goggles, and mask—proceed quickly to keep moisture out of the stock chemical.

13-sugar standard

Thoroughly dry 30 mg of each of the following monosaccharides for several days over an effective desiccant (e.g., phosphorus pentoxide): erythritol (i-erythritol, meso-erythritol), 2-deoxy-D-ribose, L(+)-rhamnose, α-D(+)-fucose (6-deoxy-D-galactose), D(−)-ribose, L(+)-arabinose, D(+)-xylose, 2-deoxy-D-glucose, β-D-allose, D(+)-mannose, D(+)-galactose, α-D (+)-glucose, *myo*-inositol. Weigh exactly 20 mg of the dried sugars into individual vials and add 1 ml Milli-Q-purified water to each. Mix together equal volumes (e.g., 100 µl) of each of the 13 monosaccharide solutions. Store the vials of 13 sugar standard at −20°C.

Sulfuric acid, 72% (w/w)

Place 20 g of ice-cold water in a beaker and add ice-cold analytical grade sulfuric acid with stirring to bring the weight to 93.5 g. Add more ice-cold water, with stirring, to bring the final weight to 100 g. Store in a glass bottle with a Teflon-lined cap.

Extreme care is necessary, wear goggles, gloves, and protective clothing. Make up the acid in a fume hood.

Trifluoroacetic acid, 2 M

Use high-purity TFA. Preparation will depend on specifications of the product purchased. For example: for TFA density 1.480: assay 99.5%; mol. wt. 114.02, 1 liter of liquid contains ~1472.6 g TFA; thus, 1 ml contains 1.4726 g TFA (or 0.679 ml/g) and 2 M TFA contains 28.04 g/liter.

To prepare 100 ml of 2 M TFA, carefully add 15.5 ml of the concentrated TFA to a 100-ml volumetric flask containing water, then adjust the volume to 100 ml with water. Store in a brown glass bottle, flush with argon or nitrogen gas, and cap tightly with a Teflon-lined lid.

CAUTION: *Extreme care is necessary; wear goggles, gloves, and protective clothing. Make up the acid in a fume hood.*

COMMENTARY

Background Information

Plant cell wall polysaccharides are composed of varying proportions of the neutral monosaccharides rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose, and the acidic monosaccharides galacturonic acid, glucuronic acid, and 4-*O*-methylglucuronic acid (see Fig. E3.2.1). Other monosaccharides have been identified but are present only in trace amounts. The monosaccharides are linked by a variety of glycosidic linkages with differing susceptibility to acid hydrolysis (Aspinall, 1982). Moreover, the monosaccharides released show variable susceptibility to degradation in acid. While procedures may be designed to achieve maximum hydrolysis, they are often a compromise to minimize destruction of the monosaccharides (Aspinall, 1982). An alterna-

tive to acid hydrolysis is to use commercially available hydrolytic enzymes. These are often highly specific for a particular glycosidic linkage and are especially suitable for detailed analysis of polysaccharide composition.

Traditionally, polysaccharides were hydrolyzed with a strong acid, such as 1 M or 2 M hydrochloric acid for 1 hr in a boiling water bath (Adams, 1965). However, some polysaccharides are completely hydrolyzed at lower acid concentrations or shorter times, and others require more strenuous conditions. Concomitantly, the monosaccharides formed by acid hydrolysis may be degraded by the acid (Aspinall, 1982). Consequently, for each polysaccharide, the investigator should vary the conditions to find the parameters that give the maximum level of hydrolysis with the minimum degradation. With a mixture of polysaccharides, finding the optimal conditions is more of a challenge and a compromise is the inevitable outcome.

Albersheim et al. (1967) made a major breakthrough when they substituted trifluoroacetic acid (TFA) for HCl or H_2SO_4. TFA is easily removed by evaporation, whereas HCl and H_2SO_4 have to be neutralized on completion of the hydrolysis. Soluble salts formed had to be removed by ion-exchange resins, with

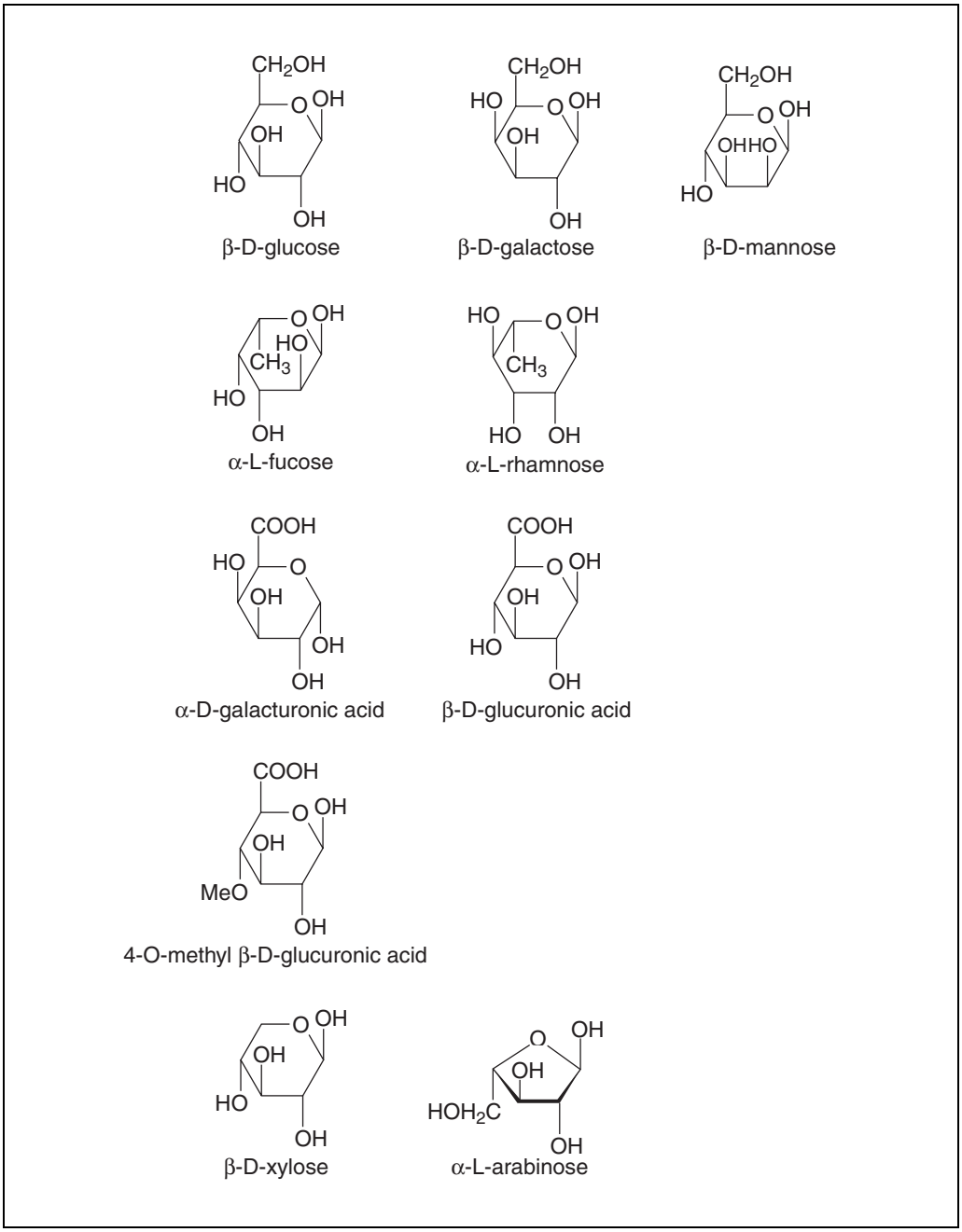

Figure E3.2.1 Structures of monosaccharides from plant cell walls.

attendant handling losses of monosaccharides. It was common to neutralize H_2SO_4 by forming the insoluble Ba_2SO_4 salt, but this adsorbs sugars, resulting in poor recoveries. However, polysaccharides containing uronic acids are resistant to acid hydrolysis, and the TFA hydrolysis procedure (Albersheim et al., 1967; Fry, 1988) gives reduced yields of the monosaccharide. Furthermore neutral sugars, such as rhamnose, are incompletely hydrolyzed if they have a uronic acid residue glycosidically linked to them. Thus, protocols described in this unit will give an estimate of the neutral monosaccharides, including the neutral side chains of the pectic polysaccharide rhamnogalacturonan, but the yield of rhamnose may be slightly underestimated. For estimation of uronic acid content see *UNIT E3.3.*

A second important breakthrough was in the acetylation step. With 1-methylimidazole as the catalyst, complete acetylation can be achieved without removing the borate formed from sodium borohydride in the prior reduction step (Blakeney et al., 1983; Harris et al., 1988). Borate complexes with *cis*-diol groups in the monosaccharides. In earlier methods (Albersheim et al., 1967; Selvendran et al., 1979), the borate had to be removed or acetylation would not go to completion (Wolfram and Thompson, 1963). The reduction and acetylation reactions are summarized in Figure E3.2.2.

Table E3.2.1 Response Factors of Alditol Acetates

Order of elution	Alditol acetate	Retention time (min)	Peak area	Response factor relative to allitol hexaacetate
1	Erythritol triacetate	14.321	1108.54	0.880
2	2-Deoxyribitol tetraaceate	18.279	1095.77	0.870
3	Rhamnitol pentaacetate	18.937	1015.50	0.806
4	Fucitol pentaacetate	19.593	1186.82	0.942
5	Ribitol pentaacetate	22.743	1191.66	0.946
6	Arabinitol pentaacetate	23.520	1195.11	0.949
7	Xylitol pentaacetate	27.075	1180.65	0.937
8	2-Deoxyglucitol hexaacetate	28.265	1214.87	0.965
9	Allitol hexaacetate	30.493	1259.49	1.000
10	Mannitol hexaacetate	31.887	1259.48	1.000
11	Galactitol hexaacetate	33.218	1254.58	0.996
12	Glucitol hexaacetate	34.828	1224.28	0.972
13	*Myo*-inositol hexaacetate	36.923	1135.60	0.902

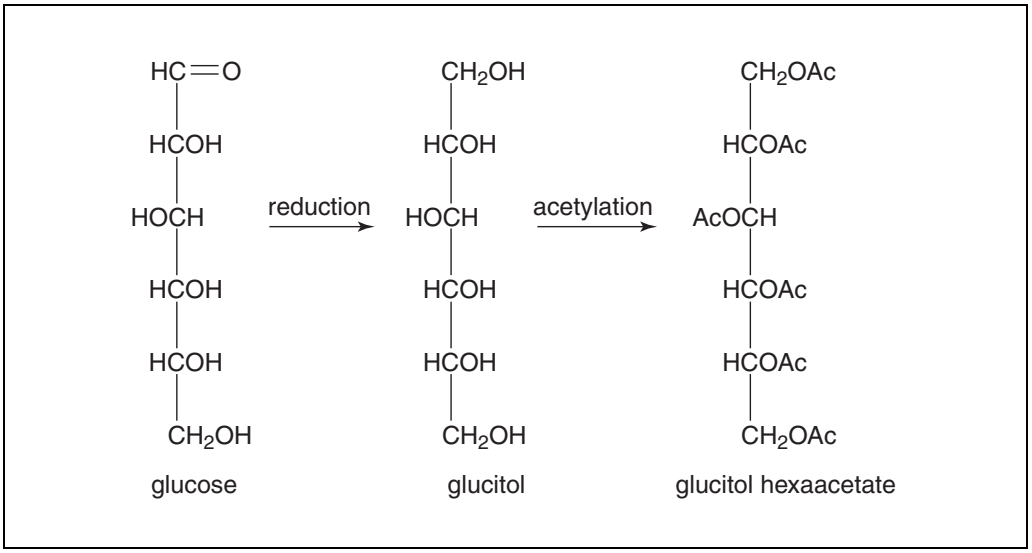

Figure E3.2.2 Reaction scheme for formation of alditol acetates.

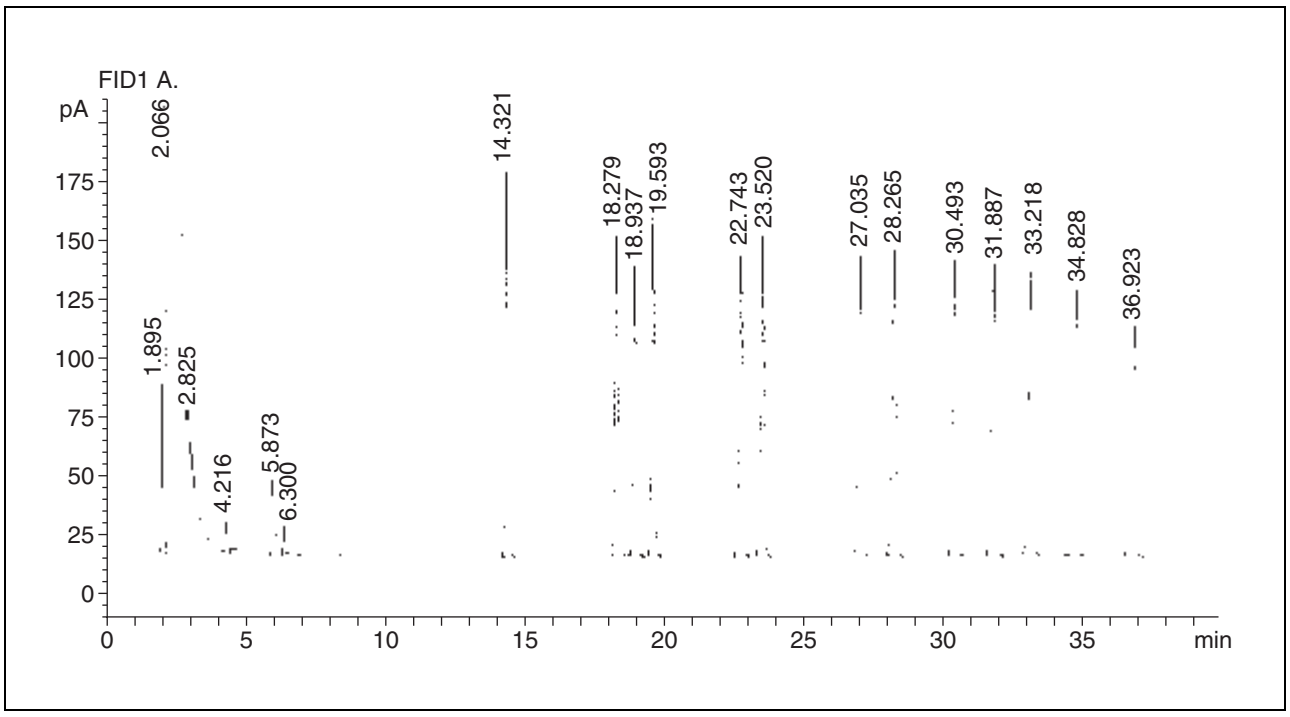

Figure E3.2.3 Chromatogram of a 13-sugar standard.

Critical Parameters

Scrupulously cleaned glassware is essential. Glassware that has previously been used for alditol acetates should first be washed in dichloromethane (DCM) to remove any traces of alditol acetates. Tubes should then be washed using high-quality detergent, rinsed at least 6 times in hot tap water, and then at least two times in distilled water, and allowed to dry.

When performing these protocols, the highest-quality analytical reagents should be used.

The syringe used to inject samples into the gas chromatograph should be washed at least ten times in fresh DCM, making sure that the outside of the needle is equally as clean as the inside. Cleaning in methanol may also assist.

Use only caps and lids that have Teflon-lined inserts.

A chromatogram from a 13-sugar standard is shown in Figure E3.2.3. The retention times, peak areas, and the response factors (i.e., allose = 1.000) are also shown. The response factors for the other monosaccharides are usually just above or below 1.000 (Table E3.2.1). The authors have found the response factor for rhamnose to be consistently lower than the other monosaccharides.

It is useful at the start of each session on the gas chromatograph to inject 0.5 µl DCM to ensure the baseline is flat and that there are no extraneous compounds eluting from the column. It is also useful to do this when one has inadvertently injected a sample that is too concentrated. Once it has been established that there are no compounds eluting, then dilute the sample further in DCM and rerun.

Troubleshooting

Some of the alditol acetates formed from deoxy sugars, pentoses, and tetroses are particularly volatile, and care should be taken when evaporating to dryness (see Basic Protocol 1, step 21). It is best to stop the evaporation as soon as the sample is dry.

Make sure exact dry weights of samples are recorded, to avoid errors associated with weighing. Special care is needed in humid environments, as cell walls and fractions readily absorb water.

Check volume of solutions in 121°C incubation at 30 min to see if tubes are leaking.

All efforts need to be made to reduce the likelihood of plasticizers (Henry et al., 1983) and other contaminants getting into one's samples. Avoid DCM making contact with the glue that attaches Teflon liners to caps, as this glue can give rise to peaks in the chromatogram.

$NaBH_4$ is degraded by reaction with moisture in the air. Therefore it should be kept in a desiccator. To check that $NaBH_4$ is reactive, take a drop of $NaBH_4$ solution and add one small drop of glacial acetic acid, which should

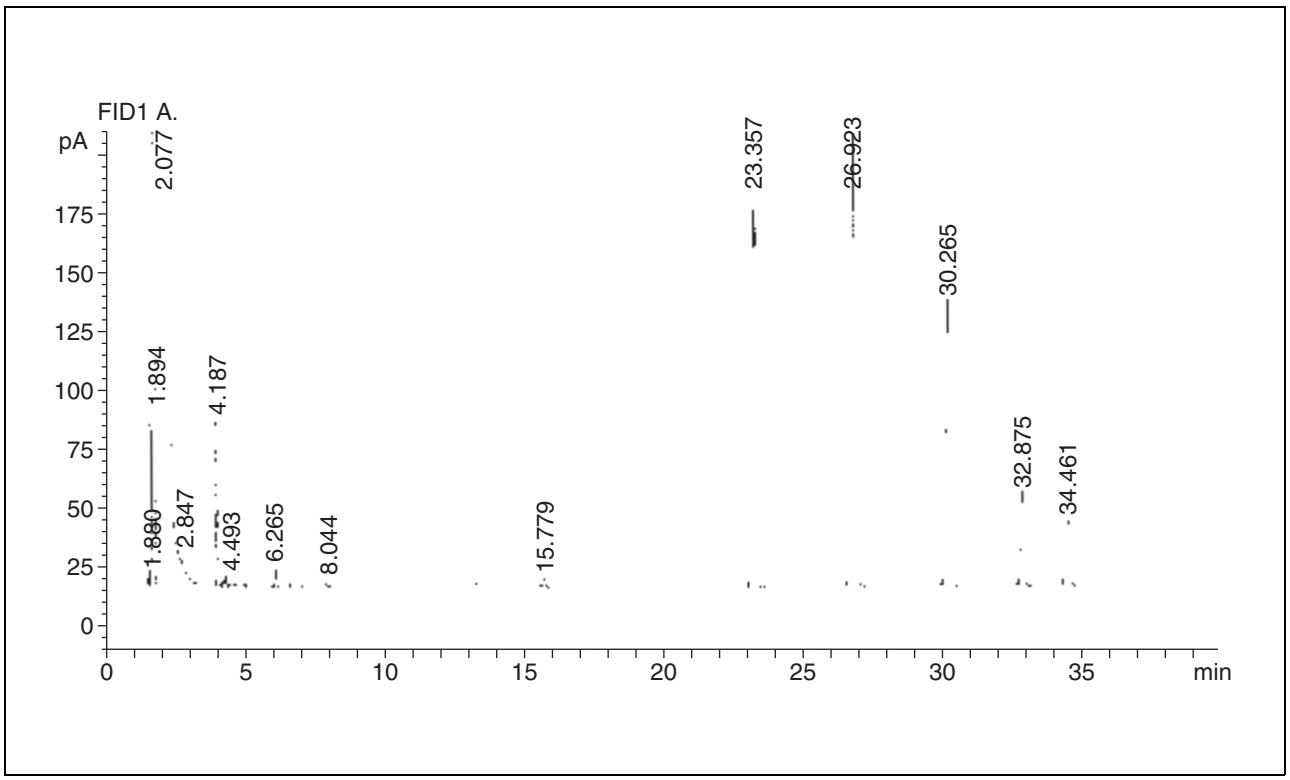

Figure E3.2.4 Chromatogram of a sample.

result in copious effervescence. Check again at the end of the reduction that there is still some unreacted $NaBH_4$ present.

Anticipated Results

Reproducibility between duplicate samples should be very high.

A sample chromatogram from a TFA hydrolysate of a cereal grain cell wall preparation is shown in Figure E3.2.4. The major monosaccharides in this sample are xylose and arabinose, with smaller amounts of galactose and glucose. The monosaccharide composition, together with the method of hydrolysis, indicate that the major polysaccharide is likely to be arabinoxylans. The glucose could arise from several different polysaccharides, including xyloglucans and (1→3,1→4)-β-D-glucans since this sample was from a cereal grain. The galactose is possibly pectic in origin. It is important when one is speculating on the types of polysaccharides in one's samples to take into account the uronic acid composition (see *UNIT E3.3*). More definitive identification requires analysis of the linkages.

When identifying the alditol acetates in a sample by comparison with the retention times of the 13-sugar standards, the times should be within 0.05 min. However, during the course of the day the retention times tend to change a little, and so it is important to rerun the 13-sugar standard after every fourth or fifth sample. Over a longer time, one will also observe slight differences in retention times, such as shown in Figures E3.2.3 and E3.2.4 for a sample and standard run 2 months apart.

Time Considerations

Do not try to process too many samples in a batch. It is suggested that initially two samples should be tried in duplicate, with an acid control, water control, and 13-sugar standard. Handling too many tubes may lead to delays during the evaporation process. Evaporation of TFA can be assisted by warming the samples to 30°C.

For Basic Protocol 1 allow 3 to 4 hr to complete steps 1 to 7. Allow a further 5 hr for steps 8 to 22. For gas chromatography allow ~50 min per sample. For Basic Protocol 2 add several more hours to that required for Basic Protocol 1, to allow for the extra hydrolysis step. The Alternate Protocol can be completed in ~8 hr.

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Key References

Albersheim et al., 1967. See above.

Describes the basic procedure for TFA hydrolysis and how the resulting monosaccharides are degraded at different rates.

Blakeney et al., 1983. See above.

Describes the procedure for reduction of monosaccharides using $NaBH_4$ in DMSO solution followed by acetylation using 1-methylimidazole as the catalyst.

Harris et al., 1988. See above.

Describes the procedure and its application to neutral detergent fiber preparations from a wide range of plants.

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Determination of the Uronic Acid Content of Plant Cell Walls Using a Colorimetric Assay

BASIC PROTOCOL

Uronic acid in the form of galacturonic acid is a major component of the pectic polysaccharide rhamnogalacturonan, which is present in large amounts in the cell walls of most fruits and vegetables. Small amounts of glucuronic acid and 4-*O*-methylglucuronic acid have also been detected in cell walls.

Quantitative measurement of total uronic acid is commonly done using colorimetric methods after first hydrolyzing the polysaccharides in sulfuric acid (Ahmed and Labavitch, 1977; Selvendran et al., 1979). However, there is a major problem with the older methods of determining uronic acid content of cell walls. Neutral sugars and their degradation products from acid hydrolysis can interfere in the colorimetric determination of uronic acids. The procedure developed by Filisetti-Cozzi and Carpita (1991) solves the problem, allowing uronic acids to be determined in up to ten times their weight of neutral sugars. For the determination of neutral sugars see *UNIT E3.2*.

The procedure can be used to determine mannuronic and iduronic acids. With glucuronic acid as the standard, the protocol could be used to measure the glucuronic acid contents of xanthan and gellan gums.

Cell walls and cell wall fractions are first hydrolyzed using the method of Ahmed and Labavitch (1977) and the uronic acid content estimated using the method of Filisetti-Cozzi and Carpita (1991).

Materials

Dried cell walls or cell wall fractions (see *UNIT E3.1*)
Concentrated sulfuric acid
4 M sulfamic acid/potassium sulfamate solution, pH 1.6 (see recipe)
75 mM sodium tetraborate (see recipe)
m-hydroxydiphenyl solution (see recipe)
0.5% (w/v) sodium hydroxide (see recipe)
D-galacturonic acid standards (see recipe)

Borosilicate glass tubes and caps
10-ml volumetric flasks
15-ml centrifuge tubes
15-ml borosilicate glass tubes
100°C water bath
Spectrophotometer

CAUTION: The uronic acid assay involves the use of concentrated sulfuric acid. Wear goggles, gloves, and protective clothing.

Hydrolyze cell walls or cell wall fractions

1. Weigh 5 mg (record exact weight) of cell walls or cell-wall fraction (in duplicate) into borosilicate glass tubes (see *UNIT E3.1* for cell wall preparation and cell-wall fractionation).

2. Add 1 ml concentrated sulfuric acid to all the tubes and cap. Set up a reagent control tube containing only 1 ml concentrated sulfuric acid and carry this through all the procedures.

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3. Place the tubes in an ice bath and stir the contents for 5 min. Do this by placing small spin bars in each of the tubes and placing the rack of tubes in a container of ice slurry on a magnetic stirrer (take care not to splatter the solid material above the level of the liquid).
4. Add another 1 ml concentrated sulfuric acid to all the tubes and stir on ice for 5 min (take care not to splatter the solid material above the level of the liquid).
5. Add 0.5 ml water and stir for 5 min on ice.
6. Add another 0.5 ml water and stir for 5 min on ice.
7. Dilute the contents of each tube with water to 10 ml in a 10-ml volumetric flask. Transfer to a 15-ml centrifuge tube and centrifuge for 10 min at 2000 × *g*, room temperature, to pellet any unhydrolyzed material.

Perform colorimetric assay

8. For each hydrolysate, set up three 15-ml borosilicate glass tubes. For the reagent control, set up two tubes. Take aliquots of 400 µl from each hydrolysate supernatant and reagent control and place in the respective tubes.
9. Add 40 µl of 4 M sulfamic acid/potassium sulfamate solution, pH 1.6, to all the tubes. Vortex contents of the tubes.
10. Add 2.4 ml of 75 mM sodium tetraborate in sulfuric acid solution to all the tubes. Vortex vigorously.
11. Place the tubes in a 100°C water bath (boiling) for 20 min, then cool by plunging tubes into an ice bath for 10 min.

    Place glass marbles on top of the tubes to prevent contamination of the samples by condensation.

12. Add 80 µl *m*-hydroxydiphenyl solution to 2 tubes of each sample and the 2 reagent control tubes. Add to the third tube of each sample 80 µl of 0.5% NaOH (this is the sample control). Vortex the contents of the tubes three times; ensure they are mixed well.

    A pink color develops within 5 to 10 min and is stable for ~1 hr after which it fades.

13. Between 10 min and 1 hr after complete mixture, read the absorbances at 525 nm against the reagent control. Subtract the values for the sample controls from their corresponding sample absorbances.
14. Prepare a D-galacturonic acid standard curve with each batch of samples.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

D-Galacturonic acid standards

Thoroughly dry D-galacturonic acid over a desiccant (e.g., phosphorus pentoxide). Make up a stock standard solution and generate a standard curve from this. Prepare a stock solution of 20 mg/ml of galacturonic acid by weighing 20 mg of the dried D-galacturonic acid into a vial and adding 1 ml of water to dissolve (this stock solution can be stored frozen). Then prepare a 200 µg/ml solution by taking 100 µl of the stock solution and adding 9900 µl of water. Use this solution to prepare the dilution series in Table E3.3.1.

CAUTION: *Phosphorus pentoxide dessicant is corrosive; wear goggles, gloves, and protective clothing.*

continued

Table E3.3.1 Dilution Series for the D-Galacturonic Acid Standard

Concentration of galacturonic acid (µg/400 µl)	Volume of 200 µg/ml galacturonic acid solution (µl)	Volume of water (µl)
5.0	125	1875
10.0	250	1750
15.0	375	1625
20.0	500	1500
30.0	750	1250
40.0	1000	1000

It may be necessary to prepare an alternative dilution series depending on the uronic acid content of your samples.

Store the stock solution frozen and prepare fresh dilutions from this for each batch.

m-Hydroxydiphenyl solution

Weigh out 0.15 g of 3-phenylphenol into a 100-ml volumetric flask, dissolve in <100 ml of 0.5% (w/v) sodium hydroxide (see recipe) then adjust the final volume to 100 ml with 0.5% sodium hydroxide. Store in a dark bottle (or wrap the bottle in aluminum foil) at 4°C. The solution is stable for ~1 month.

Sodium hydroxide, 5% (w/v)

Place 0.5 g of NaOH into a 100-ml volumetric flask. Add 20 ml water to dissolve the pellets and then adjust the volume to 100 ml with water. Prepared fresh.

Sodium tetraborate solution, 75 mM

To prepare 100 ml of 75 mM solution, weigh out 1.501 g of sodium tetraborate (mol. wt., 201.2) into a 100-ml volumetric flask and add ~90 ml concentrated sulfuric acid, and place a stopper in the flask. Stir until dissolved then adjust the final volume to 100 ml with sulfuric acid.

Ensure the sodium tetraborate is completely dissolved in the sulfuric acid. This usually requires stirring overnight. For preparation wear goggles, gloves, and protective clothing. Prepare only sufficient amount for immediate use. Keep at room temperature.

Sulfamic acid/potassium sulfamate solution, 4 M (pH 1.6)

Weigh out 38.84 g of sulfamic acid (mol. wt., 97.09) and stir vigorously in 50 ml of water. Add saturated KOH dropwise until the sulfamic acid has dissolved. Allow the sulfamic acid solution to cool and then carefully adjust the pH to 1.6 with saturated KOH. Adjust the volume to 100 ml with water to give a final concentration of 4 M. Store at room temperature.

COMMENTARY

Background Information

Uronic acids were traditionally measured by the carbazole reaction. In this procedure, sugars were treated with concentrated sulfuric or hydrochloric acid to yield products that reacted with carbazole. For example, Dische (1947) used carbazole for the determination of uronic acid in solutions of sulfuric acid, but the method was subject to interference from neutral sugars that were also present in the solution. The method was subsequently modified by Gregory (1960) and Bitter and Muir (1962) by the inclusion of borate in the sulfuric acid mixture together with a heating step. This procedure gave an increased color production but did not overcome the problem of interference by neutral sugars. A further modification was made by Galambos (1967) in which sulfamate was incorporated into the mixture prior to the heating step. Addition of sulfamate overcame the

interference problem, but the sulfamate was inclined to precipitate out; therefore, further modifications were required to deal with this. A slightly different approach was made by Blumenkrantz and Asboe-Hansen (1973). They still had sodium tetraborate in sulfuric acid, but had *m*-hydroxydiphenyl as the color reagent instead of carbazole. Unfortunately, this method was also susceptible to interference from neutral sugars. A major breakthrough in the determination of uronic acid was made by Filisetti-Cozzi and Carpita (1991) who retained the use of sodium tetraborate in sulfuric acid and *m*-hydroxydiphenyl as the color reagent but incorporated sulfamate into the mixture. The Filisetti-Cozzi and Carpita (1991) method is now the colorimetric method of choice because it can determine uronic acids in the presence of up to 10 times the weight of neutral sugars. The sulfamate suppresses the formation of brown pigments from the neutral sugars and tetraborate increases the sensitivity of the reaction with uronic acids.

Critical Parameters

Accurate pipetting of small volumes is essential for reproducibility. The absorbances should be read within 1 hr; the color fades beyond this time.

Troubleshooting

Take care to scrupulously clean glassware, as lint and other material will be degraded by concentrated sulfuric acid.

Ensure that there are no bubbles in the cuvettes when reading the absorbances. Hold the cuvettes up to the light and check before placing them in the spectrophotometer.

Ensure that there are no spillages down the sides of the cuvettes as the sulfuric acid is very corrosive and may damage equipment.

If a large excess of neutral sugars is present or browning is observed, determine the amount of neutral sugars present (*UNIT E3.2*) in the sample. Record the absorbance spectrum (400 to 700 nm) of the corresponding amount of neutral sugars and subtract it from the spectrum of the colorimetric reaction.

The presence of other polysaccharide gums that contain uronic acids (alginates, xanthan, and gellan) causes serious interference.

Anticipated Results

Careful technique should result in reliable results with high reproducibility. Absorbances of replicate samples should be within 0.005 units.

Time Considerations

Allow a few days to prepare for this assay. The D-galacturonic acid standard has to be dried over a desiccant for several days and the sodium tetraborate requires stirring overnight to dissolve in sulfuric acid.

The time to complete this assay will depend on the number of samples one does in a batch. For the first attempt, it is suggested that only two samples be analyzed along with the standard curve. This will probably take ~4 hr. Although it is tempting to do large numbers in a batch, it does take time to read them all on the spectrophotometer, especially if only read one at a time, and within the hour.

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Key Reference

Filisetti-Cozzi and Carpita, 1991. See above.

An excellent account of method development.

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Determining the Degree of Methylation and Acetylation of Pectin

BASIC PROTOCOL

Pectins from plant cell walls generally consist of a backbone of galacturonic acid residues interspersed with rhamnose residues to which side chains of arabinans, galactans, and arabinogalactans are attached. In addition, the galacturonic acid residues can be methyl esterified at C6 and acetylated at the O2 or O3 positions (Figure E3.4.1). In the food industry, knowledge of the degree of methylation and acetylation of pectins is useful because the extent of methylation and acetylation affects the gelling properties of the pectin; a higher degree of methylation increases the capacity to form gels, whereas a higher degree of acetylation inhibits gelling (Whistler and BeMiller, 1997).

The degree of methylation of pectins can be estimated by determining the proportion of methyl esters in the pectin sample in conjunction with the total uronic acid content. This unit describes the method of Voragen et al. (1986) for determination of the extent of methylation. The advantage of this method is that the degree of acetylation is determined simultaneously. For this procedure an HPLC system fitted with a refractive index detector is necessary. In order to determine the degree of esterification, the total uronic content must be known. The total uronic acid content can be measured quantitatively using the colorimetric procedure of Filisetti-Cozzi and Carpita (1991) as described in *UNIT E3.3*.

Materials

Plant material sample
Isopropanol/NaOH solution: 50% (v/v) isopropanol/0.4 M NaOH, 4°C
5 mM H_2SO_4 (made fresh, sterilized with a 0.45-μm filter, and degassed)
Methanol and acetic acid standards (see recipe)

Centrifuge tubes fitted with gas-tight caps or lids, smallest available size (e.g., microcentrifuge tubes)
Small vials with septa, smallest available size (e.g., 15-ml autosampler vials for HPLC)
High-performance liquid chromatography (HPLC) system with refractive index detector and HPLC column (e.g., Aminex HPX-87H, Bio-Rad) fitted with suitable guard column

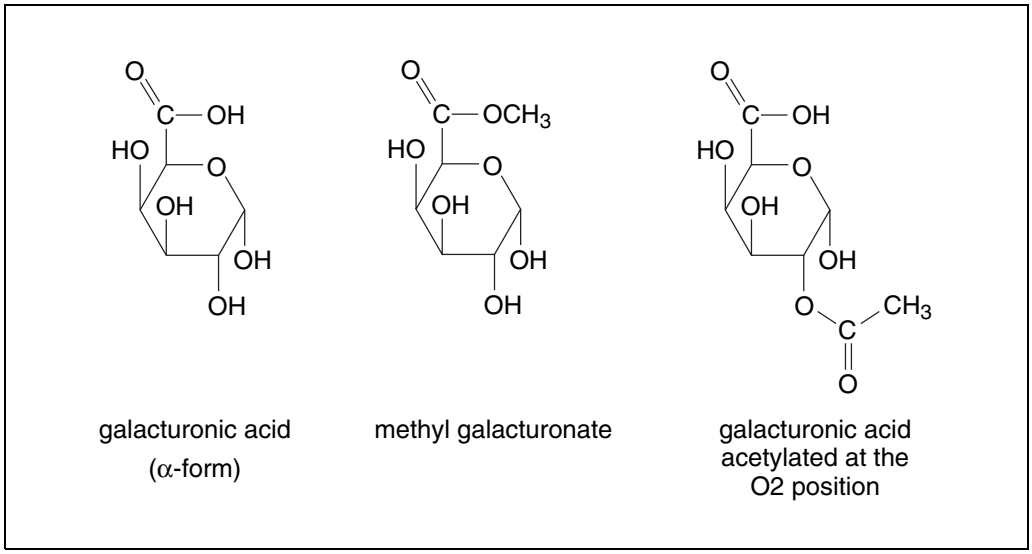

Figure E3.4.1 Structure of galacturonic acid showing methyl esterified and acetylated forms.

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Additional reagents and equipment for isolating pectin (*UNIT E3.1*) and determining total uronic acid content (*UNIT E3.3*)

CAUTION: Chemical substances such as sodium hydroxide and sulfuric acid require special handling; see *APPENDIX 2B* for guidelines.

Prepare sample

1. Isolate cell walls from a plant material sample using an appropriate cell wall isolation protocol (*UNIT E3.1*).
2. Fractionate cell wall polysaccharides with *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) to obtain pectin-containing polysaccharide fractions (*UNIT E3.1*).

   Fractions containing pectin are likely to be those obtained from isolated cell walls following treatment with CDTA. Fractions obtained following treatment with solutions of alkali are not suitable (see Troubleshooting). Alternatively, commercial preparations of pectin can be analyzed.

3. Determine total uronic acid content from an aliquot of each fraction using the method described in *UNIT E3.3*.

Treat with alkali

4. Weigh duplicate ~30-mg samples of pectin (noting actual weight) and place each into a centrifuge tube fitted with gas-tight cap or lid.
5. Set up a control tube containing no pectin and carry this through the procedure.

   This control is analyzed to ensure there is no contamination by methanol or acetic acid. If methanol or acetic acid is detected in the HPLC trace from this tube, the experiment should be repeated.

6. Add 1 ml isopropanol/NaOH solution (4°C) to each tube. Cap and mix gently.

   The solid material must not be splattered above the level of the liquid.

   The tubes must be capped at all times as methanol and acetic acid are volatile.

7. Allow mixture to stand 2 hr at room temperature.
8. Centrifuge 10 min at 2,000 × *g*, at room temperature.
9. Immediately remove supernatant and place in a small vial with a septum. Seal vial immediately.

   The supernatant must be removed immediately as the phases will otherwise remix.

Measure methylation and acetylation

10. Remove 15 µl clear supernatant, preferably through the septum (or alternatively using an HPLC automatic injection device), and inject into an HPLC system.
11. Use a 5 mM H_2SO_4 solvent system set at a flow rate of 0.6 ml/min and a temperature of 30°C. Set a refractive index detector at 40°C.
12. Use 15-µl aliquots of methanol and acetic acid standards (0 to 5 mg of each) injected into the HPLC system to prepare a standard curve.
13. Plot area from HPLC trace (response area) on the *y* axis versus concentration of methanol or acetic acid (in milligrams per milliliter) on the *x* axis.
14. Identify methanol and acetic acid peak in sample chromatogram by comparison with retention time for appropriate standard (see Anticipated Results).

15. Calculate amount of methanol and acetic acid in the sample (in milligrams per milliliter) from sampler response area using standard curve.

16. Calculate degree of methyl esterification using the following formula:

    Degree of methylation = (millimoles methanol/millimoles uronic acid) × 100

    For a sample calculation, a 30-mg sample was found to have 30.1% uronic acid content (UNIT E3.3). Therefore, it had 9.03 mg or 0.051 mmol uronic acid. (The molecular weight or uronic acid is 176 g/mol.) The amount of methanol in this sample, as calculated from the standard curve, was 1.2 mg/ml. Therefore, in 1 ml of sample in isopropanol/NaOH, there would be 1.2 mg or 0.0375 mmol methanol. (The molecular weight of methanol is 32.04 g/mol.) Therefore, the degree of methylation = (0.0375/0.051) × 100 = 74%.

17. Calculate degree of acetylation in the same way.

    The molecular weight of acetic acid is 60.05 g/mol.

    It is possible for the degree of acetylation to be >100% (e.g., Renard and Jarvis, 1999), whereas the degree of methylation has a maximum value of 100%.

REAGENTS AND SOLUTIONS

*Use analytical-grade reagents and HPLC-grade water in all recipes and protocol steps. For suppliers, see **SUPPLIERS APPENDIX**.*

Methanol and acetic acid standards

Prepare 100 mg/ml methanol solution by accurately weighing 1 g methanol into a clean, dry 10-ml volumetric flask. Bring to volume with chilled (4°C) isopropanol/NaOH (see Basic Protocol). Repeat for acetic acid, but transfer weighed acid into chilled isopropanol/NaOH, taking care to rinse the weighing dish well with further isopropanol/NaOH (to avoid losses), and adjust volume to 10 ml. Prepare 10 mg/ml solutions by pipetting 1 ml of each 100 mg/ml solution into separate clean, dry 10-ml volumetric flasks and then bringing to volume with chilled isopropanol/NaOH. Prepare standard solutions as shown in Table E3.4.1 by first pipetting out the volumes of the chilled isopropanol/NaOH and then adding the appropriate volumes of methanol or acetic acid solutions. Immediately cap tubes and swirl to mix. Prepare fresh solution for each analysis.

CAUTION: *When working with glacial acetic acid, work in a fume hood and wear safety glasses, gloves, and protective clothing.*

Using chilled isopropanol/NaOH will prevent loss of volatile methanol and acetic acid.

Table E3.4.1 Preparation of Methanol and Acetic Acid Standards

Concentration (mg/ml)	Isopropanol/NaOH (µl)	10 mg/ml solution (µl)
0.0	1000	0
1.0	900	100
2.0	800	200
3.0	700	300
4.0	600	400
5.0	500	500

COMMENTARY

Background Information

Methods of measuring the degree of methylation of pectins include the titration of pectin with polycations (Mizote et al., 1975) and retitration of the increase of carboxyl groups following saponification (e.g., Schultz, 1965b). However, for pectins that are highly acetylated, saponification methods can give an erroneously high value. In such situations the acetyl content must be determined separately (Schulz, 1965a) and an adjustment made. One alternative that does not suffer interference from acetyl groups is the copper binding method, which is based on the difference in copper complexing with the pectin before and after saponification (Keijbets and Pilnik, 1974). The copper binding method is seldom used nowadays, probably because of difficulties with interfering compounds, an inherent aspect of such a method. Other procedures for measuring the degree of methylation depend on deesterification by saponification to yield methanol. The methanol released is then measured colorimetrically after oxidation to formaldehyde and reaction with a chromophore (Filippov and Kuz′minov, 1971; Wood and Siddiqui, 1971). Alternatively, the methanol is measured by gas chromatography (McFeeters and Armstrong, 1984) or by gas chromatography after conversion to methylnitrite (Litchman and Upton, 1972).

The method of Voragen et al. (1986), in which treatment with alkali is followed by measurement of methanol and acetic acid (Figure E3.4.2), is attractive because of its simplicity and speed. Refractive index detection is a reliable method for quantifying the compounds after separation by HPLC. Furthermore, the degree of acetylation is determined simultaneously. However, with all these methods except for the copper binding method, the total uronic acid content, a measure of galacturonic acid in the pectin sample, must be determined separately, and thus the final result is influenced by the accuracy of this estimation. The authors have discussed the problems of total uronic acid determination in *UNIT E3.3* and recommend the method of Filisetti-Cozzi and Carpita (1991).

Prior to measurement of the degree of methylation and acetylation, the pectin must be extracted from the cell walls. Methods for isolating and fractionating cell walls are described in *UNIT E3.1*. The procedure can, of course, be used on commercial pectin samples.

Critical Parameters

This protocol can be used for determining the extent of methylation and acetylation of pectin in fractions obtained following CDTA treatment of isolated cell walls (*UNIT E3.1*). It can also be applied to a commercial pectin. In principle the method could be used on whole cell walls, but the authors advise caution for the following reasons. Whole cell walls are much more complex than a polysaccharide fraction, and complete hydrolysis may be more difficult to achieve. Moreover, in a mixed system such as the cell wall, only a "total" extent of methylation or acetylation could be reported, as sub-

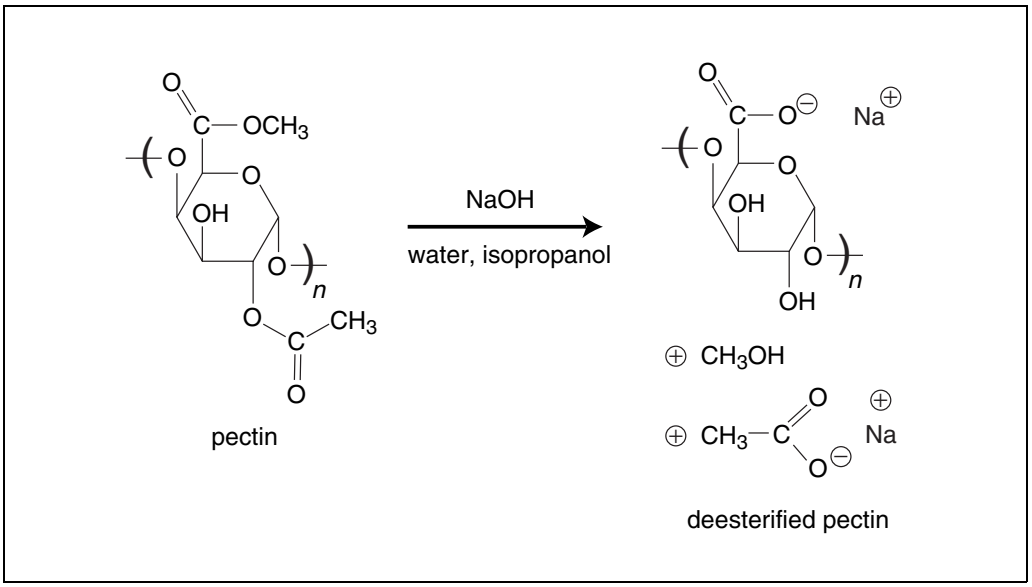

Figure E3.4.2 Principle of the reaction. Methanol (CH_3OH) and acetic acid (CH_3COOH) dissolve in the isopropanol/NaOH solution while the pectin (both esterified and deesterified) is insoluble.

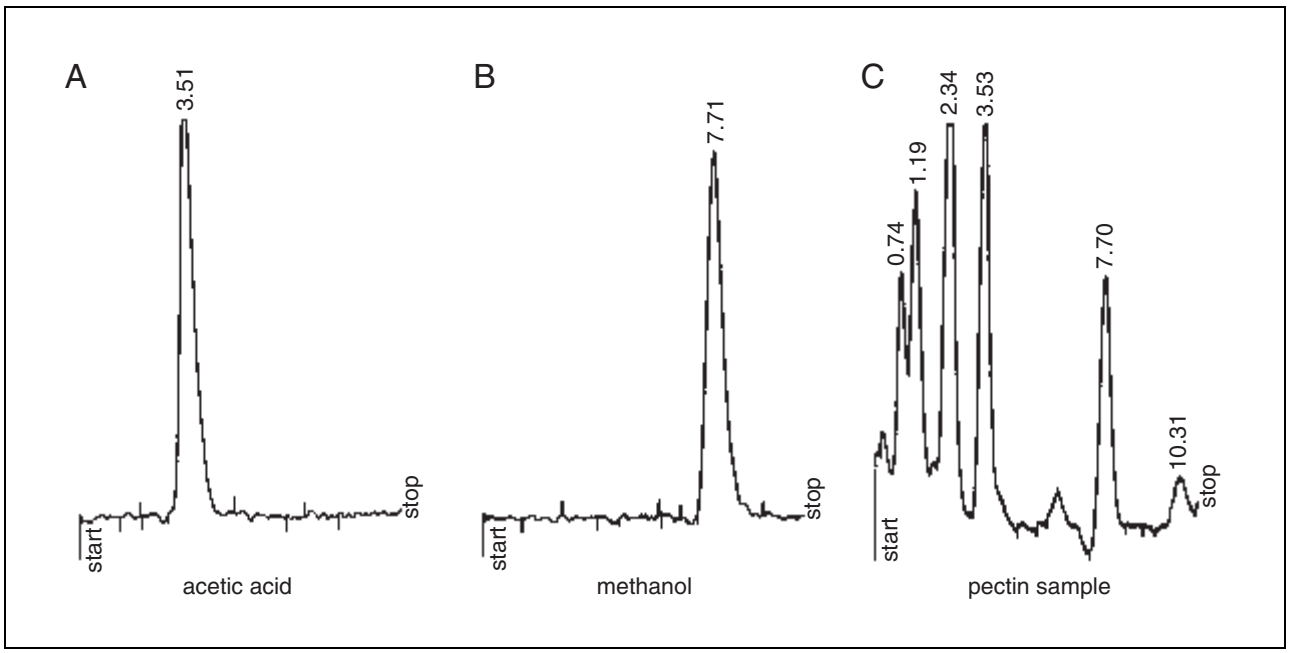

Figure E3.4.3 Representative chromatograms. (**A**) Acetic acid standard (0.4 mg). (**B**) Methanol standard (2.0 mg). (**C**) Pectin sample. The start of each recording begins 12 min into HPLC analysis. Numbers above each peak refer to minutes elapsed after start of recording. The additional peaks in (C) are presumably due to other alcohols and acids released by alkaline treatment.

Table E3.4.2 Degree of Methylation and Acetylation of Some Pectins

Source	Methylation (%)	Acetylation[a] (%)	Reference
Apple	80	6	Renard and Thibault (1993)
Commercial apple pectin	70-72	3-4	Voragen et al. (1986)
Commercial citrus pectin	64	2	Voragen et al. (1986)
Kiwifruit (at harvest)	71	ND	Redgwell et al. (1990)
Kiwifruit (ripe)	49	ND	Redgwell et al. (1990)
Nectarines (at harvest)	80	ND	Dawson et al. (1992)
Nectarines (ripe)	68	ND	Dawson et al. (1992)
Mango	68	4	Voragen et al. (1986)
Pear	13	14	Voragen et al. (1986)
Potato	31	14	Voragen et al. (1986)
Sugar beet	21	3	Voragen et al. (1986)

[a]ND, not determined

stituents on polymers other than pectin, such as acetyl groups on xylans, are likely to be hydrolyzed. Thus, the authors recommend that analyses be performed on pectin fractions as described in the above protocols, while remembering that fractions obtained following treatment with solutions of alkali cannot be analyzed, as the methyl and acetyl substituents have already been removed by this treatment (Figure E3.4.2).

Centrifuge tubes with gas-tight caps or lids should be used in this experiment. It is important to keep the tubes capped at all times as methanol and acetic acid are volatile. To further prevent loss of these volatiles, the isopropanol/NaOH solution should be chilled before adding the volatile methanol and acetic acid when preparing the standards.

Troubleshooting

As treatment with alkali causes decarboxylation, this method cannot be used on material that has been fractionated using alkaline solutions (*UNIT E3.1*).

The underlying assumption for this protocol is that all the methoxyl and acetyl groups are

attached to the galacturonic acid residues. If for any reason they are not, inaccurate results will be produced. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy of the pectin solution can help clarify the positions where methoxyl and acetyl groups are attached to the pectin (Keenan et al., 1985).

Anticipated Results

Good reproducibility can be expected. Figure E3.4.3 shows chromatograms of acetic acid and methanol standards and a pectin sample. Additional peaks are observed in the HPLC traces (Figure E3.4.3C). These are presumably due to other alcohols and acids (e.g., ferulic acid; Renard and Thibault, 1993) released by alkaline treatment. Table E3.4.2 shows the degrees of methylation and acetylation of pectin samples.

Time Considerations

Allow a full working day the first time this protocol is carried out to give sufficient time for preparing standard solutions, completing the procedure (note that the mixture must stand for 2 hr in step 7), and performing HPLC.

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Filisetti-Cozzi and Carpita, 1991. See above.

Excellent account of the method for colorimetric determination of total uronic acid.

Voragen et al., 1986. See above.

Describes the background and methodology for the protocol and shows elution profiles for standards and a sample.

Contributed by Laurence D. Melton
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Quantitative Determination of β-Glucan Content

Mixed linkage β-glucan is the polymer of (1→3)(1→4)-β-D-glucose and is a constituent of barley and oat endosperm cell walls. Beside cereals, it is also found in some kinds of lichens. β-Glucan has been shown to have health-promoting properties such as reduction of serum cholesterol, regulation of blood glucose, and immunostimulation. Based on successful clinical trials, the U.S. Food and Drug Administration (FDA, 1997) approved a health claim for oat products containing 0.75 g of β-glucan per serving, indicating that it reduces the risk of heart disease. Therefore, accurate determination of the actual amount of β-glucan in raw materials and final food products became increasingly important. The enzymatic method of b-glucan determination is based on the procedure of McCleary and Glennie-Holmes (1985) and a subsequent, more-streamlined procedure developed by McCleary and Codd (1991). The latter method uses smaller sample sizes (80 to 120 mg) compared to the standard procedure (500 mg) as well as a lower dilution, which increases the chance for error, especially if a material with a high concentration of β-glucan is analyzed (i.e., >50%). More recently, the streamlined procedure was developed into official methods of analysis (AACC, 1995; AOAC Int., 2000). Both methods recommend a sample size of only 80 to 100 mg. To accommodate a large variety of samples and novice analysts, the standard Megazyme procedure (Megazyme, 2001; *http://www.megazyme.com*), which uses a sample size of 450 to 500 mg flour or <50 mg β-glucan gum, is recommended by the authors. The main focus of the method presented here (see Basic Protocol) is on the analysis of cereal flours and β-glucan concentrates isolated from barley and oats. Alternate Protocols 1 to 4 are proposed for several types of processed food products. Procedural modifications essentially deal with sample preparation; the β-glucan quantitation steps are almost identical to those described in the Basic Protocol.

ANALYSIS OF β-GLUCAN IN CEREAL FLOURS AND β-GLUCAN CONCENTRATES

BASIC PROTOCOL

This protocol is adapted from the Megazyme Mixed Linkage β-Glucan Assay kit developed by Dr. McCleary (Megazyme International Ireland, 2001). It uses highly specific enzymes for (1→3)(1→4)-β-glucan hydrolysis without affecting other polysaccharides of glucose such as starch and cellulose; however, the original procedure has been modified to increase β-glucan detectability, which became a significant problem with the new high β-glucan cultivars of barley and oats that exhibit increased grain hardness. These cultivars give higher proportions of coarse particles upon milling, resulting in the underestimation of β-glucan content. Sample preparation in this procedure is modified to accommodate such samples.

Materials

Sample: cereal grain or meal, or β-glucan gum or concentrate
50% (v/v) ethanol
20 mM sodium phosphate buffer, pH 6.5 (see recipe)
Mixed linkage β-glucan assay kit (Megazyme):
  50 U/ml lichenase solution (see recipe)
  2 U/ml β-glucosidase solution (see recipe)
  GOPOD reagent (see recipe)
50 mM sodium acetate buffer, pH 4.0 (see recipe)
1 mg/ml glucose standard solution, 4°C (Sigma; see Critical Parameters and Troubleshooting)

Contributed by Zvonko Burkus and Feral Temelli
Current Protocols in Food Analytical Chemistry (2003) E3.5.1-E3.5.12

Laboratory mill (e.g., UDY Cyclone Sample Mill) equipped with 250-µm screen
Analytical balance with 0.1 mg precision
Leak-proof centrifuge tubes with caps (see recipe)
Multipipettor with 5- and 50-ml Combitips (no. 1 on dial = 100 µl or 1 ml, respectively) or equivalent
40° to 45°C, 45° to 50°C, and boiling water baths
~6- to 12-ml round-bottom glass test tubes or vials with screw caps
Spectrophotometer with 1-ml quartz cuvette
Aluminum container (optional)
80°C oven (optional)

Additional reagents and equipment for determining moisture content (optional; see Chapter A1)

Prepare sample

1. If starting with a previously ground meal, homogenize bulk material or sample before regrinding (see Critical Parameters and Troubleshooting).

   Flour obtained through pin-milling tends to become stratified with differing levels of β-glucan when placed in a bucket.

2. Process sample in a laboratory mill equipped with a 250-µm screen and transfer to a capped container. Homogenize resulting flour by stirring, mixing, and tumbling to ensure uniform distribution of coarser β-glucan-rich particles prior to taking a representative sample for analysis.

   Do not use a 500-µm screen as recommended by Megazyme.

3. Using at least duplicate samples, accurately weigh ≤500.0 mg ground material on a balance with a precision of 0.1 mg by placing the sample directly on the bottom of leak-proof centrifuge tubes with caps. For barley and oat cultivars rich in β-glucan (up to 10%), reduce the sample size so that the sample absorbance reading will not be more than that for the 1 mg/ml glucose standard.

   For barley or bran with 10% to 20% β-glucan, sample size should be only 150 to 300 mg. For materials with a higher level of β-glucan (50% to 90%), reduce the sample size to ~38 to 45 mg (e.g., for a β-glucan gum containing >70% β-glucan). If analyzing a β-glucan gum or concentrate, make sure that there are no lumps and that the sample is free-flowing and resembles flour or grits.

   To avoid loss of material, never use a pan or paper for weighing and transferring sample to tubes.

4. Add 1 ml of 50% ethanol to the tube, cap the tube, and vortex vigorously. Leave 1 min to allow ethanol to penetrate sample.

5. Load a multipipettor and 50-ml Combitip with 20 mM sodium phosphate buffer, pH 6.5, and set the dial to 5. Expel all air from the tip as would be done with a medical syringe. Add 5 ml buffer to each tube. Cap the tubes and immediately vortex vigorously, ensuring that the top of the vortex is below the tube neck. Use a glass rod to disperse any visible lumps.

6. Heat the sample 1 to 2 min in a boiling water bath and vortex vigorously so that no lumps are visible. Tighten tube caps if necessary. Repeat for a total of 6 min heating time. If sample is too thick, add 4 to 5 ml distilled water.

   Total heating time should be at least 6 min, as a shorter time is not sufficient to dissolve all β-glucan. If necessary, heating time can be extended to 8 to 9 min. An 8- to 9-min heating time may result in a somewhat higher sample blank for free glucose, but will significantly improve β-glucan recovery, especially if the sample was ground through a 500-µm screen.

7. Equilibrate sample by swirling in a 40° to 45°C water bath (1 min for glass or 2 min for plastic).

   Do not immerse tubes in an ice water bath since they may crack.

Perform β-glucan hydrolysis

8. Add 200 µl lichenase solution (10 U) using a multipipettor set to 2 and a 5-ml Combitip. Cap immediately and vortex.

   An alternative dispenser may be used, even a simple micropipet, but a multipipettor saves time.

   If the sample is very viscous, add 4 ml water at this point, but decrease water in step 10 so that the total amount of water added remains constant.

9. Incubate 60 min at 45° to 50°C, vortexing every 15 min. Ensure that there is no material stuck to the tube wall. If necessary, wash the sides by increasing vortex speed.

   β-Glucan solubility increases with temperature, and lichenase works very well even at 50°C.

10. Add 24 ml distilled water (total volume 30.2 ml). Cap the tubes and vortex vigorously at least 10 sec.

11. Centrifuge the tubes (or a 1- to 5-ml aliquot from each sample) 10 to 12 min at 1000 × *g*, room temperature, to precipitate particulates.

    Higher centrifugal force may be used to save time (e.g., 5 min at 2000 × g), but a further increase in centrifugal force may result in cracked tubes.

    If a centrifuge is not available, particulates can be removed by filtration through Whatman no. 41 filter paper. This method, however, may lead to significant underestimation of β-glucan content.

12. Using a 100-µl micropipettor with disposable plastic tips, transfer 100 µl supernatant (or filtrate) from each duplicate sample to the bottom of three ~6- to 12-ml test tubes or vials with caps.

    If the pipettor does not deliver exactly 100 µl (check by weighing an aliquot of water; see Critical Parameters and Troubleshooting), it should not affect the final result since the same amount will be used in all three portions. The same micropipettor must be used for the glucose standard and samples. Do not use oversized tips (e.g., 500 or 1000 µl), since this leads to increased error. Precise pipetting is critical for accuracy of results (see Critical Parameters and Troubleshooting).

13. To the first tube (sample blank), add 100 µl of 50 mM sodium acetate buffer, pH 4.0, using a multipipettor set at 1 and a clean 5-ml Combitip. Add 100 µl β-glucosidase solution (0.2 U) to the two other tubes. Cap immediately. Gently vortex or swirl to mix enzyme and sample.

    Note that as samples are prepared in duplicate (step 3) there should be two blanks and four sample tubes at this step.

    There is a risk that with a vigorous vortexing, a few or even a single drop (sample is only 200 µl) may splash to the lip or upper walls of the tube and be lost for color reaction. Thus, gentle vortexing is absolutely necessary.

14. Incubate 15 min in a 45° to 50°C water bath or oven.

Prepare glucose standard

15. Pour a small amount (≤1 ml) of 1 mg/ml glucose standard into a clean capped vial. Cap and return the stock solution to the refrigerator immediately. Allow the aliquot to reach room temperature (~10 min).

    For accurate quantification, it is essential that the glucose standard be equilibrated to room temperature before an aliquot is taken for measurement (step 16), and that aliquots be taken using the most accurate pipetting techniques. For further details, see Critical Parameters and Troubleshooting for discussions on glucose standards and pipetting technique.

16. Prepare three tubes or vials. Combine 100 µl each distilled water and 50 mM sodium acetate buffer into the first tube (reagent blank). Transfer 100 µl of the prewarmed glucose standard into the bottom of the other two test tubes and cap them. Discard any remaining prewarmed standard solution.

    The same precision in pipetting is also required for the glucose standard. To avoid microbial contamination, never return surplus glucose standard to the stock bottle.

Perform color development

17. Add 3 ml GOPOD reagent to the bottom of each test tube using a positive-displacement pipet (e.g., multipipettor set at 3 and 50-ml Combitip). Expel all the air from the Combitip before dispensing GOPOD. Cap the tubes and vortex gently. Return GOPOD to the refrigerator immediately.

18. Incubate capped vials 20 min at 45° to 50°C.

19. Bring samples to room temperature. Vortex or swirl tubes to rinse the condensate from the tube walls.

    Samples can be stored up to 2 hr wrapped in aluminum foil before measurements are taken.

Determine free glucose

20. Measure absorbance of reagent blank at 510 nm in a 1-ml quartz cuvette and zero the spectrophotometer. Next, measure absorbance of the sample blank, sample solutions, and standards.

    Click only once to adjust to zero when using the reagent blank, because spectrophotometers usually adjust themselves again after a second click. Newer spectrophotometers should adjust to zero automatically. Use only one clean 1-ml quartz cuvette with two opaque walls. If the spectrophotometer has a flow-through cell, make sure that it is cleaned regularly. For further details, see Critical Parameters and Troubleshooting.

21. Calculate the percentage β-glucan in the sample (fresh weight or dry weight) according to the equation:

$$\% \beta\text{-glucan} = (A_S - A_B) \times W_G/A_G \times 302 \times 100/W_S \times 162/180$$

$$= (A_S - A_B) \times 2718/(W_S \times A_G)$$

where A_S is the average absorbance of the sample, A_B is the absorbance of the reagent blank, W_G is the weight of glucose in 100 µl of glucose standard (0.1 mg), A_G is the average absorbance of the glucose standard, 302 is the dilution factor (0.1 ml sample taken from 30.2 ml), 100 is the factor for calculating percent, W_S is the weight of the sample, and 162/180 is a factor for water bound to polymerized glucose through hydrolysis.

Sample weight (W_S) can be either fresh weight or dried weight in mg. If % moisture is not known, proceed with step 22. If fresh and dry weights and % moisture are known, skip to step 24.

22. Determine percentage moisture content (see Chapter A1). Alternatively, if sample is limited, accurately weigh 0.5 to 2 g sample into an aluminum container and dry 20 hr at 80°C. Cool in a desiccator containing recently reactivated absorbent and weigh again. Calculate moisture content as:

    % moisture = (wet weight – dry weight) × 100/wet weight.

23. Calculate β-glucan content in the sample on a dry matter (dm) basis according to the equation:

    % β-glucan (dm basis) = % β-glucan (fresh weight) × 100/(100 – % moisture).

24. Calculate free glucose from the equation:

    $\%\ \text{glucose} = A_B \times W_G/A_G \times 302 \times 100/W_S$

    $= A_B \times 3020/(A_G \times W_S)$

    In this case, the factor 162/180 is omitted because the glucose is free glucose.

    If the same sample is to be used for starch analysis, this calculation can be used for the determination of free glucose (UNIT E2.2), and the ethanol wash can be avoided. Consequently, the sample blank in this analysis can also serve as a sample blank for starch analysis.

ANALYSIS OF β-GLUCAN IN BREADS, MUFFINS, AND OTHER BAKED PRODUCTS

ALTERNATE PROTOCOL 1

Breads or muffins enriched in β-glucan may be produced using oat or barley flour or bran, as well as coarser particles such as oat flakes or cracked barley. Coarser particles tend not to disperse uniformly in a dough. Therefore, it is important to obtain a representative sample from such products in order to have uniformly distributed β-glucan-rich particles.

Additional Materials *(also see Basic Protocol)*

Baked food sample (e.g., bread, muffins)
60° to 80°C oven

1. Use at least two nonadjacent slices of bread per loaf from three loaves. Do not use end slices. Cut three muffins into four squares and take two pieces from opposite sides of each muffin. Immediately record the weight of each sample.
2. Dry overnight at 60° to 80°C (depending on thickness).

    A lower drying temperature is desirable. Bread or muffin pieces can be cut into crumbs to enhance the drying rate.

3. Leave dried pieces exposed to ambient air for 48 hr to stabilize moisture content.
4. Record the dried weight.
5. Proceed with β-glucan determination (see Basic Protocol, steps 3 to 24).
6. Recalculate β-glucan content in fresh bread or muffins from the formula:

    β-glucan in fresh bread = β-glucan in dried bread × (dried weight/fresh weight)

    Final result for fresh products must be lower than that for dried products.

ANALYSIS OF β-GLUCAN IN PASTA PRODUCTS

β-Glucan in dry pasta products can be determined using the Basic Protocol. Since pasta particles tend to be firmer than other cereal products, cooking time may be extended if necessary (up to 15 min; see Basic Protocol, step 6). Fresh pasta products require additional sample preparation steps. It can be analyzed in either pureed or dried form. In ready-to-eat meals, β-glucan may be incorporated into a side dish such as pasta or pot barley. Because it will most likely be mixed with a sauce, which will interfere with β-glucan determination, the pasta must be washed prior to analysis.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Fresh pasta sample
Kitchen blender (e.g., Waring Blendor; optional)

Puree method

1a. Homogenize fresh pasta sample to a smooth puree in a kitchen blender. If necessary, use equal amounts of pasta and water, but record the weight of each before homogenizing.

2a. Take ~1 g pasta puree and proceed with analysis (see Basic Protocol, steps 3 and 5 to 24).

The ethanol step should be omitted.

3a. Calculate β-glucan in fresh pasta using the formula:

β-glucan in fresh pasta = β-glucan in diluted pasta × (diluted pasta weight/fresh pasta weight)

Drying method

1b. Dry fresh pasta sample as described for bread (see Alternate Protocol 1, steps 1 to 4).

2b. Proceed with analysis (see Basic Protocol, steps 3 to 24), but exclude ethanol (step 4; pasta is not too sticky) and extend cooking time by 2 to 5 min (step 6) to completely dissolve β-glucan.

3b. Recalculate β-glucan content in fresh pasta from the formula:

β-glucan in fresh pasta = β-glucan in dry pasta × (dry weight/fresh weight)

Final result for fresh products must be lower than that for dried products.

Ready-to-eat meals

1c. Place all of the side dish (pasta or pot barley) in a strainer and wash gently up to 10 sec using lukewarm (35° to 40°C) water.

2c. Spread sample on a paper towel and gently press with another paper towel to remove excess moisture from washing. Use a mild air stream to dry off any residual water.

3c. Continue with β-glucan determination by the puree method (steps 1a to 3a above).

4c. Calculate the percentage of β-glucan based on the total weight of the meal.

DETERMINATION OF β-GLUCAN IN CEREAL BARS

ALTERNATE PROTOCOL 3

For low-moisture bars such as granola bars, no special preparation is necessary except for fine milling. If added sugar presents a problem (i.e., higher glucose absorbance readings), sugars must be removed. For chewy bars with a higher moisture content and potentially a higher lipid content, it may also be necessary to dry the sample and remove lipids.

Additional Materials *(also see Basic Protocol and Alternate Protocol 1)*

Cereal bar sample
80% *or* 96% to 100% (v/v) ethanol (optional)

For high-sugar bars

1a. Mix 0.5 to 1.0 g cereal bar sample with ~10 ml of 80% ethanol and heat 5 min in an 80° to 85°C water bath.

2a. Centrifuge 10 min at 1000 × *g*, 25°C, and decant ethanol without losing sample.

3a. Repeat once and then proceed with analysis (see Basic Protocol, steps 3 to 24).

For chewy cereal bars

1b. Cut a whole cereal bar into small pieces (e.g., 1-cm cubes) and weigh. Dry as for analysis of bread (see Alternate Protocol 1, steps 2 to 4), but leave dried pieces exposed to ambient air for only ~2 hr (step 3). Record the dried weight.

Longer stabilization may allow sugars to rehydrate and become too sticky before milling.

2b. Mill finely and immediately cap the sample.

3b. Accurately measure ~0.5 to 1.0 g sample (see Basic Protocol, steps 1 to 3).

4b. Remove sugars (steps 1a to 3a above).

5b. *Optional:* Wash sample three times for 30 min with 10 ml of 96% to 100% ethanol while stirring. Decant ethanol after each wash without losing sample. If necessary, heat tubes to ~60°C.

Sugar removal (step 3b) will also remove the majority of lipids. The ethanol wash is performed when lipids still present a problem during glucose reading.

6b. Proceed with β-glucan determination (see Basic Protocol, steps 5 to 24).

DETERMINATION OF β-GLUCAN IN MALT AND SPENT GRAIN

ALTERNATE PROTOCOL 4

Analysis of malt and spent grain is performed as described in the main method (see Basic Protocol). The β-glucan content of these samples, however, is very low due to enzymatic hydrolysis during germination of barley. In addition, malting barley varieties contain less β-glucan. The amount of sample should therefore be increased to 1 g, and addition of ethanol may be omitted. A high blank reading should be expected due to hydrolyzed starch. If glucose readings are too high (much more than that for the glucose standard), remove preexisting glucose from milled samples as described in Alternate Protocol 3 (steps 1a to 3a) and continue with the Basic Protocol starting from step 5.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers see* ***SUPPLIERS APPENDIX****.*

Glucose oxidase/peroxidase/4-aminoantipyrine (GOPOD) reagent

Dilute 20× phosphate buffer (*UNIT E2.2*) to 1× using distilled water. Add a small amount to a vial of GOPOD powder (see annotation), dissolve completely, and transfer to a 1-liter volumetric flask. Rinse the vial with another portion of buffer and add to the flask. Bring the solution to the 1-liter mark with buffer. Store in the dark up to 3 months at <5°C.

GOPOD powder is supplied as >12,000 U glucose oxidase, >650 U peroxidase, and 81.3 mg 4-aminoantipyrine, and is yellowish in color. It can be purchased separately or as part of the Megazyme β-glucan assay kit.

The appropriate 20× phosphate buffer is described in UNIT E2.2, and is also supplied with the Megazyme starch determination kit. If a large number of samples is analyzed and the buffer will be used in <15 days, sodium azide can be omitted from the buffer, which is desirable since it is toxic.

CAUTION: *Use gloves when handling sodium azide, and add sodium azide after pH adjustment since acidification of azide may release a toxic gas.*

β-Glucosidase solution, 2 U/ml

Combine 1 ml of 40 U/ml purified β-glucosidase concentrate (Megazyme kit) with 19 ml of 50 mM sodium acetate buffer (see recipe) in a pasteurized 50-ml capped tube. Cap and vortex vigorously. Store frozen up to 1 year. If small amounts of enzyme are used, aliquot into 4 or 5 plastic vials and avoid freeze-thaw cycles.

Be careful not to cross-contaminate enzymes. Use clean tips for handling each enzyme.

Leak-proof centrifuge tubes with caps

Ensure 35- to 50-ml polypropylene or glass centrifuge tubes are leak proof at high temperatures by adding 5 ml water, capping, and measuring the mass. Incubate in a boiling water bath 10 min, then dry, cool, and weigh again. A weight difference of 20 mg is acceptable.

Glass tubes, if used, must not be chipped at the mouth.

Lichenase solution, 50 U/ml

Mix 1 ml of 1000 U/ml purified lichenase concentrate (Megazyme kit) with 19 ml of 20 mM sodium phosphate buffer (see recipe) in a pasteurized 50-ml tube. Cap and then vortex vigorously. Store frozen up to 1 year. If small amounts of enzyme are used, aliquot into 4 or 5 plastic vials and avoid freeze-thaw cycles.

If using glass tubes, they should be of a large volume (i.e., twice the enzyme volume) and freeze tilted at a >60° angle to prevent cracking from ice expansion.

Sodium acetate buffer, 50 mM, pH 4.0

Combine 2.9 ml glacial acetic acid with 900 to 950 ml distilled water in a clean 1-liter beaker. Stir and adjust to pH 4.0 using 1 N NaOH. Transfer to a 1-liter volumetric flask using a clean funnel and adjust volume to 1 liter with water. Store up to 1 year at <5°C.

Sodium phosphate buffer, 20 mM, pH 6.5

Precisely weigh 3.12 g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), transfer to a 1-liter beaker, and add 900 to 940 ml distilled water. Adjust pH to 6.5 using 0.1 N NaOH (usually ~50 ml). Transfer to a 1-liter volumetric flask using a clean funnel and adjust volume to 1 liter. Store up to 1 year at <5°C.

Table E3.5.1 Influence of Particle Size on the Quantification of β-Glucan in β-Glucan-Rich Oats

Raw material	Percentage (w/w) β-glucan content (dm basis)	
	Retsch 24-pin hammer-mill with 500-μm screen	UDY mill with 250-μm screen
Groat meal	6.7	7.8
Fiber concentrate	25.8	29.7

COMMENTARY

Background Information

The health claim approved for oat products (FDA, 1997) encourages food product developers to incorporate oats into various processed products to meet the requirement of 0.75 g of β-glucan originating from oat flour, groats, meal, and similar materials. Even though the health claim does not encompass oat gum or any product containing barley, there is an increasing interest in the use of oat or barley β-glucan gum as a food ingredient, based on the growing body of evidence on the health benefits of β-glucan, as well as its effects on food texture. Currently, a variety of processed food products contain oat-based ingredients (e.g., flour, bran, flakes, meal), whereas the use of barley-based ingredients is very limited. For the purpose of labeling and making a health claim, accurate determination of β-glucan in raw materials and food products is critical.

Enzymatic hydrolysis of β-glucan is carried out in two steps using two enzymes of high purity. First, lichenase cuts (1→4) bonds adjacent to (1→3) bonds, resulting in the formation of β-gluco-oligomers mostly having three to twelve glucose units. Approximately 90% of the glucose in β-glucan structure is in the form of oligomers containing three or four glucose units after lichenase treatment. Oligomers with greater than nine glucose units usually precipitate out of solution because they are in fact short cellulosic rods, which tend to associate into insoluble microcrystalline rods. Thus, it is crucial to do the second hydrolysis step as soon as possible, where β-glucosidase hydrolyses the oligomers formed in the first step into molecular glucose. Free glucose is incubated with glucose oxidase/peroxidase (GOPOD) and yields a product with a reddish color. The colorimetric reaction from glucose to the quinone-imine dye is the same as that used for enzymatic starch determination (see Figure E2.2.1). If either the lichenase or β-glucosidase used in β-glucan determination is not pure and contains traces of starch hydrolysis enzymes (i.e., α-amylase and amyloglucosidase), additional glucose will be liberated from the starch present in flour or other cereal-based products and give erroneously high β-glucan content.

Critical Parameters and Troubleshooting

Several factors are critical for the accuracy and reproducibility of β-glucan determination. As with any other analysis, some of these factors relate to the skills of the analyst. Parameters critical for β-glucan analysis include the following discussed below: sample homogeneity, particle size, enzyme purity, glucose standard, pipetting technique, and absorbance measurement.

Sample homogeneity

Having a homogeneous and representative sample for analysis is critical. If a bulk flour (e.g., 20 kg) is received in any kind of bag or pail, make sure it is homogeneous by mixing it thoroughly, and then take a sample of sufficient size for chemical analyses (e.g., 200 g). If particle size is not known, regrind the sample to ≤250 μm and homogenize again. This will ensure that the sample is representative of the bulk.

Particle size

Regardless of their nominal screen size (i.e., 500 μm), laboratory mills are different in their internal design and consequently produce meal of different granulation. Centrifugal mills, like the Retsch GmbH Ultra Centrifugal Mill ZM 100, have a screen along the whole perimeter of the milling chamber. The openings are in the form of a shredder positioned vertically against particle movement. Although openings are nominally 500 μm, shredding can result in longer particles with a substantial amount of undisrupted cells. On the other hand, the UDY Cyclone Mill has a milling chamber that

abrades particles during rotation, and the screen occupies ~1/15 of the perimeter. By the time the particles hit the screen openings, which are positioned more tangentially, the particles are substantially pulverized and the proportion of grits is much smaller. In addition, soft materials like oats often clog the screen, increasing pulverization. Therefore, milling with a UDY mill with a 500-µm screen may result in satisfactory β-glucan determination when working with soft materials, while milling using the Retsch mill may lead to a significant underestimation of β-glucan content (Table E3.5.1). For milling whole kernels using a UDY mill with a 250-µm brass screen, the kernels should first be cracked or milled using a 1-mm screen, because whole kernels may damage the 250-µm screen. Although the Retcsh mill equipped with a 500-µm screen is recommended by Megazyme, based on the above data and the experience of the authors, finer grinding using a 250-µm screen is highly recommended.

Enzyme purity

As discussed above, the purity of enzymes is critical for accurate β-glucan determination. Testing every new batch of enzymes on pure starch (e.g., wheat- or cornstarch; A.E. Staley Manufacturing) is essential, especially if a supplier other than Megazyme is used. A color reaction that shows more free glucose than that specified by the manufacturer (i.e., <0.1%) is the first sign of impure enzymes—e.g., contamination by amylase enzymes. Enzyme impurity can be further verified by extending the incubation time, which liberates more free glucose, resulting in a darker color with GOPOD. Check color development as described in the Megazyme manual and *UNIT E2.2*. Note that, as samples are boiled, enzyme inhibitors are mostly denatured and thus not a source of contamination.

Glucose standard

The 1 mg/ml glucose standard solution from Sigma (available in 100-ml bottles) is prepared using 0.1% benzoic acid. The manufacturer recommends storage at 2° to 8°C. The glucose standard should be kept refrigerated in a tightly capped bottle. For accurate measurement, a portion of the standard must be brought to room temperature before sampling, as reduced temperatures will increase the total amount of glucose measured and thus result in higher A_G readings. To ensure accurate sampling, an aliquot (i.e., ≤1 ml) should be warmed to room temperature in a capped vial or tube, and then the sample to be measured should be taken using precise pipetting technique as described below. Any remaining prewarmed standard should be discarded and not returned to the cold stock solution.

Although preserved, the glucose standard may deteriorate over time (usually >6 months). Microbial deterioration causes lower glucose readings for the standard (A_G), leading to an overestimation of β-glucan content in the sample. It is important to note that extended storage in the refrigerator may dry and concentrate the standard even when apparently tightly capped. If the glucose standard reading varies more than ±0.03 AU from the expected values, the glucose standard should be replaced. Day-to-day variability in the spectrophotometer or in fresh GOPOD reagent is very rare. It is also possible that the supplier may provide a standard of substandard quality. Verifying the absorbance reading of a glucose standard from a different supplier is a cheap solution. An alternative is to prepare a glucose standard (at least 1 liter) in house to determine anticipated absorbance readings. This value can then be considered a benchmark for the absorbance of glucose for thousands of samples. Absorbance readings for glucose standards or samples should not be too high. Values >1.20 should be avoided since the glucose standard curve is not linear in this region.

A 1 mg/ml glucose standard solution is also supplied as part of the Megazyme β-glucan kit (~5 ml), and is prepared using 0.2% benzoic acid, which is the accepted preservative for glucose solutions. Megazyme recommends storage of the glucose standard at room temperature to ensure that aliquots are measured accurately, and states that their standard has no loss of activity after prolonged storage (i.e., several years) at room temperature (B. McCleary, pers. comm.). Nonetheless, the authors of this unit prefer storage at 4°C in a tightly capped bottle, as they feel the standard may deteriorate over time (usually >6 months) at room temperature and cause erroneous β-glucan calculations.

Pipetting technique

Supervisors usually assume that novice analysts joining their group know how to use a micropipettor properly. Usually, this is not the case; therefore, some suggestions for accurate pipetting of viscous samples involved in β-glucan analysis are presented here. Do not immerse the pipet tip more than 3 mm into the solution. Deeper immersion usually leads to

adsorption of a small drop on the outer wall of the tip. Pull the sample inside the tip *slowly*. Wipe the tip on the neck of the sample tube by a circular motion, being careful not to touch the tube wall with the mouth of the tip. Push the sample out slowly while holding the micropipettor completely vertical. This will allow time for a viscous sample to come down the walls. Push out the last remains of the sample quickly (when piston stops). This will prevent the last drop from sticking to the tip. If, however, there is still a drop on the outside of the tip, touch the bottom of the test tube and wipe the sample solution. When taking 100 µl of glucose standard, do not use the first draw because it only wets the inside of the tip. Return the aliquot and take a second. With the sample, wetting is not a problem because the first pipetting of a sample is for the sample blank to determine free glucose, which is usually very low for grain samples. After the third (final) pipetting of the same sample solution, *discard the pipet tip*. Disposable pipet tips are not to be washed and reused. Finally, keep in mind that all pipet tips are not of the same quality. They are made of different materials and sometimes with a special coating, like Teflon. Before using a new batch of tips, practice pipetting into a beaker placed on an analytical balance. The weights of several 100-µl aliquots should not differ by more than 1 mg for distilled water. If a sample solution (which is usually more viscous) sticks to the inside of a tip, change to another kind of tip.

Absorbance measurement

Since the results rely on the accuracy of the absorbance measurement, it must be done properly. When turning on a spectrophotometer, wait 5 to 10 min for the lamp to stabilize. If a flow-through cell is not available, a perfectly clean quartz cuvette must be used. Check by looking through the cuvette toward a light source. If necessary, clean with ethanol and acetone. The quartz cuvette should have opaque sides and a 1-ml volume with opaque sides, since the total sample volume is only ~3 ml. Plastic cuvettes are not good for precise determinations. Wash the cuvette with distilled water only between samples. It is not necessary to dry the inside of the cuvette. Before adding the next sample, wash the cuvette walls twice with 0.5 ml (½ cuvette volume) of the next sample by tilting and rotating, then pour out the fluid and add the sample (0.6 ml may be enough since the light travels through the lower half of the cuvette in a majority of spectrophotometers). Wipe the clear walls with a lint-free tissue (e.g., Kimwipes EX-L) while holding the opaque sides. Do not touch clear sides. Place the cuvette with the arrow facing the light source so that light travels ~1 cm through the clear walls of the cuvette. Measure absorbance twice and take an average value. If the difference is more than 0.004 AU, repeat the reading. When finished measuring the third tube of the same sample, use distilled water to wash the cuvette. Then wash it with the sample blank (first light colored tube) of the next sample. If working close to a window or strong light source, cover samples with aluminum foil.

Anticipated Results

The absorbance reading for glucose standard in the authors' hands (using a Hewlett Packard 8452A Diode Array spectrophotometer) is 1.06 ± 0.02 (maximum). Even narrower spread is achievable with precise pipetting. Absorbance for glucose standard in the enzymatic analysis of starch is ~3% or ~0.03 AU higher. β-Glucan content of grains can vary substantially depending on variety and environmental conditions. In general, the β-glucan content of barley is in the range of 2% to 8% (w/w, dm basis), whereas that of oats is 2% to 7%. Some new barley varieties have been reported to have as high as 17% β-glucan. Different fiber concentrates obtained by dry or wet processing techniques can have 10% to 70% β-glucan. β-Glucan gum produced by aqueous extraction may reach >80% β-glucan, and even >95% (dm basis) after further purification. Wheat usually contains <0.7% β-glucan, rice and maize <0.3%, and rye and triticale 1% to 2.5% β-glucan. Free glucose is usually present at <0.5%. Barley and oat grains or flour stored for a long time (e.g., >1 year) may contain up to 1% free glucose. More highly processed cereal products (e.g., cookies, granola bars, ready-to-eat cereals) targeting the 0.75 g/serving recommendation may have a β-glucan content of 1.6% to 3% based on a 25- to 45-g serving size. Malt and spent grain usually contain <1% β-glucan.

Time Considerations

Assuming that all materials and enzymes are prepared ahead of time and that samples are already weighed into centrifuge tubes, it may be possible to analyze two series of twelve samples each, in duplicate, in 8 to 9 hr (without breaks). This is about the maximum that can be achieved in a day if the second series of twelve samples is started during the 60-min lichenase incubation for the first series. The time neces-

sary for clean up and calculations of results are not included. A novice analyst may expect to analyze eight to twelve samples in duplicate in a day when all glassware is ready. Thus, the total time for analysis is about 4.5 hr, plus clean up and calculations. A specialized laboratory equipped with automatic dispensers, a large-capacity centrifuge to process all samples at once, and a spectrophotometer with a flow-through cell or an automatic sampler may be able to handle a larger number of samples per day (i.e., 60 to 100 samples).

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Key Reference

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A booklet supplied with the β-glucan determination kit containing directions for standard and streamlined procedures for β-glucan determination in barley and oats, as well as the procedure for β-glucan determination in malt, wort, beer, and spent grain.

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Extraction, Isolation, and Purification of Anthocyanins

Anthocyanins are the flavonoid compounds that produce plant colors ranging from orange and red to various shades of blue and purple. This unit describes methods for extraction, isolation, and purification of anthocyanin pigments from plant tissues. These methods are essential laboratory operations prior to subsequent experimental work involving separation, characterization, and quantitation of the pigments (*UNITS F1.2 & F1.3*). The polar character of the anthocyanin molecule allows for its solubility in many different solvents such as alcohols, acetone, dimethyl sulfoxide, and water. The choice of extraction method should maximize pigment recovery with a minimal amount of adjuncts and minimal degradation or alteration of the natural state. Basic Protocol 1 describes the extraction of anthocyanins with acetone and their partition with chloroform. This procedure permits concentration of anthocyanin pigments in the aqueous phase while removing lipids, chlorophylls, and other water-insoluble compounds. The Alternate Protocol describes the extraction of anthocyanins with acidified methanolic solutions. Methanol is the most commonly used solvent for anthocyanin extraction because its low boiling point allows for rapid concentration of the extracted material. However, the resultant extract contains low-polarity contaminants and further purification may be necessary. Basic Protocol 2 describes a simple, fast, and effective method for purification of anthocyanins from polyphenolic compounds, sugars, and organic acids using solid-phase adsorption. The Support Protocol describes a method for preparing a finely powdered sample using liquid nitrogen. This produces a uniform composite sample with a high surface area, which allows for efficient pigment extraction.

ACETONE EXTRACTION AND CHLOROFORM PARTITION OF ANTHOCYANINS

BASIC PROTOCOL 1

In this method, acetone extracts the anthocyanins from the plant material, and chloroform partitioning further isolates and partially purifies the pigments. The addition of chloroform results in phase separation between the aqueous portion (which contains the anthocyanin, phenolics, sugars, organic acids, and other water-soluble compounds) and the bulk phase (which contains the immiscible organic solvents, lipids, carotenoids, chlorophyll pigments, and other nonpolar compounds). This method has the advantage of producing an extract with no lipophilic contaminants. The absence of a concentration step minimizes the risk of acid-dependent pigment degradation.

Materials

Powdered plant material (see Support Protocol), frozen
Acetone
70% (v/v) aqueous acetone *or* aqueous acidified acetone: 70% aqueous acetone with 0.01% HCl
Chloroform
Acidified water: 0.01% (v/v) HCl in deionized, distilled water
Waring Blender with stainless steel container (Waring) *or* general-purpose homogenizer

Whatman no. 1 filter paper
Buchner funnel
Separatory funnel
500-ml boiling flask
Rotary evaporator with vacuum pump or water aspirator, 40°C

Contributed by Luis E. Rodriguez-Saona and Ronald E. Wrolstad
Current Protocols in Food Analytical Chemistry (2001) F1.1.1-F1.1.11

CAUTION: Chloroform is a toxic irritant and mild carcinogen; take suitable precautions (*APPENDIX 2B*) and work in a fume hood.

1. Mix ~50 g powdered plant material (accurately weighed and recorded) 1:1 (w/v) with acetone using a Waring Blender with stainless steel container or a general-purpose homogenizer.

   For most materials, a 1:1 ratio of sample to solvent should be used. For materials rich in pectic substances, however, a higher proportion of acetone may be required, e.g., 1:1.4 or 1:2.

   For dried samples or materials with high sugar content, the sample needs to be dispersed in water before extracting with acetone. A general guildine is to suspend dried sample in the amount of water that would be present in the fresh tissue. If a blender is used, it should be explosion proof.

   Alternatively, the plant material and acetone can be mixed with a chemical-resistant stir-bar.

2. Separate the anthocyanin extract (filtrate) from insoluble plant material by filtering the slurry through a Whatman no. 1 filter paper by vacuum suction using a Buchner funnel.

3. Reextract plant material with 70% (v/v) aqueous acetone until a clear or faintly colored solution is obtained. If plant material has a pH ≥4, use aqueous acidified acetone. Pool filtrates and discard plant material.

   Three subsequent extractions should be sufficient.

   The use of acidified acetone ensures that the aqueous fraction will be at a low pH where the anthocyanins are more stable, and that the chloroform/acetone solvents will be in an acidic environment.

   In the authors' experience, the pigments of a wide variety of plant materials have been stable in the aqueous acetone extract. However, problems have been encountered with pigment degradation for certain potato cultivars with high polyphenol oxidase activity. This problem can be circumvented by putting the aqueous acetone extract (uncapped) in a boiling water bath for 5 min. After heating, the volume of acetone lost by evaporation should be replenished. This enzyme inactivation step has been found unnecessary for most materials.

4. Transfer filtrate to a separatory funnel, add 2 vol chloroform, and gently mix by turning funnel upside down a few times. Store sample overnight at 4°C or until a clear partition between the two phases is obtained.

   CAUTION: *Chloroform/acetone is explosive if it comes in contact with strong alkali. Waste solvent should be stored in a labeled container strictly used for disposal of these solvents, and not mixed with other materials. Disposal is conducted according to implemented safety guidelines of the institution. If there is a possibility of the waste turning alkaline, some hydrochloric acid should be added to ensure it remains acidic.*

   For small sample sizes, a 1:1 ratio of filtrate to chloroform can be placed in a screw-cap test tube, mixed, and centrifuged to separate the phases. Rapid and clean phase separation is produced with this method.

5. Transfer the aqueous phase (upper portion) to a 500-ml boiling flask. Remove residual acetone/chloroform in a rotary evaporator at 40°C under vacuum.

   The presence of anthocyanin pigments in the aqueous phase is evidenced by the pink to red, purple, or blue color of the solution.

The evaporating flask should be less than one-half full for efficient solvent removal. Generally, the flask volume should be four to five times that of the solvent volume. Prolonged evaporation time should be avoided to minimize pigment degradation. Evaporation should be complete in 5 to 10 min.

6. Make up remaining aqueous extract to a known volume (usually 100 ml) with acidified deionized distilled water. If the sample is to be analyzed within 2 days, store extract at 4°C. For longer periods (up to 1 year or even longer), store at −18°C. Avoid repeated freezing and thawing.

METHANOL EXTRACTION OF ANTHOCYANINS

ALTERNATE PROTOCOL

This is the classical method of extracting anthocyanins from plant materials. This procedure involves maceration or soaking of the plant material in methanol containing a small concentration of mineral acid (e.g., HCl). Methanol extraction is a rapid, easy, and efficient method for anthocyanin extraction. However, a crude aqueous extract with several contaminants is obtained, and methanol evaporation can result in hydrolysis of labile acyl linkages, which is aggravated by the presence of HCl.

Additional Materials *(also see Basic Protocol 1)*

Acidified methanol: 0.01% (v/v) HCl in methanol

1. Homogenize 50 g powdered plant material (accurately weighed and recorded) in 2 vol (w/v) acidified methanol. Allow it to macerate 1 hr.

   One hour should be sufficient time for anthocyanin extraction because of the high surface area of the powdered material. Materials are often allowed to extract overnight under refrigerated conditions, particularly when materials have been ground directly in the blender with acidified methanol and not previously powdered.

2. Filter slurry through a Whatman no. 1 filter paper by vacuum suction using a Buchner funnel.
3. Reextract plant material with acidified methanol until a faint-colored extract is obtained. Pool filtrates and discard plant material.

   Three subsequent extractions should be sufficient.

4. Transfer filtrates to a boiling flask and evaporate methanol in a rotary evaporator at 40°C under vacuum.

   The evaporating flask should be less than one-half full for efficient solvent removal. Prolonged evaporation time should be avoided to minimize pigment degradation. A marked reduction in the rate of evaporation as well as an apparent increase in viscosity in indicative that the residual liquid is mostly water.

   If pigment isolate is to be analyzed in an aqueous system, the extract should not be taken to dryness. For some analytical applications, the sample should be taken to dryness and then dissolved in methanol or other appropriate solvent. In the latter concentration stages, azeotropic removal of water can be facilitated by addition of methanol.

5. Make up remaining aqueous extract to a known volume with acidified deionized distilled water, water, methanol, or other appropriate solvent. If the sample is to be analyzed within 2 days, store extract at 4°C. For longer periods (up to 1 year or even longer), store at −18°C. Avoid repeated freezing and thawing.

   Extract should be made up in acidified water or water if continuing with Basic Protocol 2.

SUPPORT PROTOCOL

SAMPLE PREPARATION FOR ANTHOCYANIN PURIFICATION

The plant material is frozen with liquid nitrogen and powdered using a Waring Blender or mill suitable for use under extremely low temperatures. The use of liquid nitrogen minimizes anthocyanin degradation by lowering the temperature and providing a nitrogen environment. The fine powder maximizes pigment recoveries due to its high surface area and favors disruption of cellular compartments.

Materials

Liquid nitrogen
Plant material

Waring Blender with stainless steel container (Waring), M 20 IKA-Universal Mill (IKA Works), *or* equivalent
Teflon or stainless steel container (or equivalent container able to withstand −210°C)
Freeze-resistant container, high-density polyethylene (HDPE) or equivalent

1. Pour liquid nitrogen into a dry stainless steel Waring Blender container or M 20 IKA-Universal Mill grinding chamber and allow to evaporate. If operating a blender, turn on for few seconds. Repeat this procedure until container is well chilled.

   CAUTION: *Wear suitable protective goggles and gloves when pouring liquid nitrogen.*

2. Freeze 50 g plant material with liquid nitrogen in a separate container of Teflon, stainless steel, or other material that can withstand temperatures of −210°C.

   For most plant materials, a representative sample size of 50 g should allow for a reliable estimate of the anthocyanin content; however, if the amount of material is limited, the method can be applied to a minimum sample of 1 g.

   Most berries and seeds can be frozen as is, but other plant materials of larger size should be reduced in particle size to ~2 cm^3 with a stainless steel knife.

3. Blend frozen plant material using the chilled container (step 1).

4. Open blender or mill, add liquid nitrogen, allow to evaporate, and repeat grinding process until a fine powder is obtained.

   CAUTION: *The blender or mill lid should not be tightly closed until liquid nitrogen has evaporated completely to avoid explosive separation of the lid. Lids can be modified with a venting stainless steel tube or chimney to avoid this hazard.*

5. Transfer powdered material to a freeze-resistant container for analysis, or store it immediately at −20°C.

   Some powdered materials are hygroscopic, making accurate weighing (see Basic Protocol 1 or Alternate Protocol) difficult. For quantitative analyses, it may be advisable to weigh fresh material (before freezing), and quantitatively transfer powdered material using an appropriate solvent.

BASIC PROTOCOL 2

ANTHOCYANIN PURIFICATION

Purification of anthocyanin-containing extracts is often necessary, as the solvent systems commonly used for extraction are not specific for anthocyanins. Considerable amounts of accompanying materials may be extracted and concentrated in the colored extracts, which can influence the stability and/or analysis of these pigments (Jackman and Smith, 1996). Anthocyanin purification using solid-phase extraction (Figure F1.1.1) permits the removal of several interfering compounds present in the crude extracts. Mini-columns containing C_{18} chains bonded on silica retain hydrophobic organic compounds (e.g.,

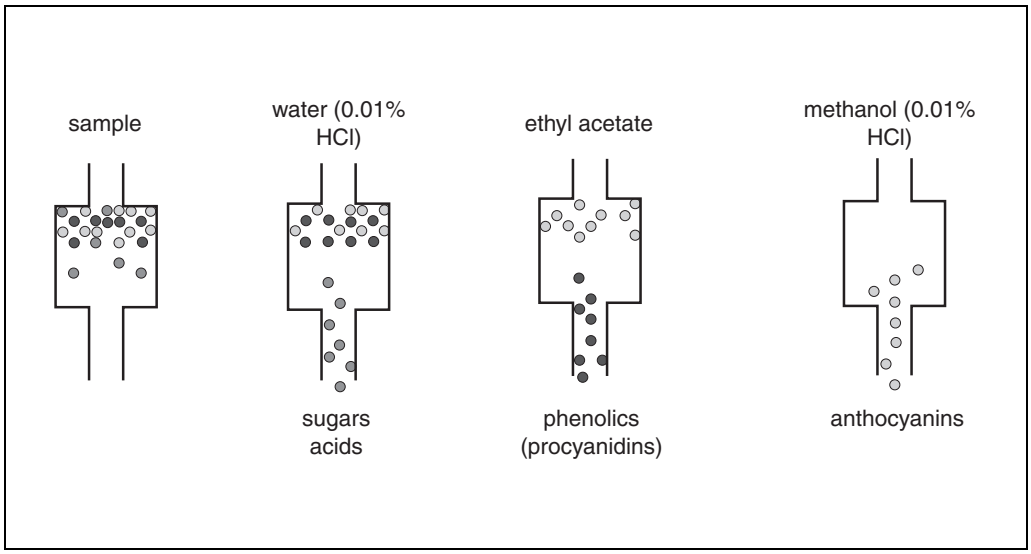


Figure F1.1.1 Solid-phase (C18) purification of anthocyanins. The sample components (represented by differentially shaded circles) are resolved by subsequent wash steps as indicated. The last wash, with acidified methanol, elutes anthocyanins. Acidified methanol and/or water should be used as solvents if electrospray mass spectrometry is to be carried out subsequently.

anthocyanins, phenolics), while allowing matrix interferences such as sugars and acids to pass through to waste. Washing the retained pigments with ethyl acetate will further remove phenolic compounds other than anthocyanins.

Materials

Methanol
Acidified water: 0.01% (v/v) HCl in deionized, distilled water
Aqueous anthocyanin extract (see Basic Protocol 1; see Alternate Protocol)
Ethyl acetate
Acidified methanol: 0.01% (v/v) HCl in methanol

C18 cartridge (with C_{18} sorbent bonded on silica: C_{18} Sep-Pak Cartidge (360 mg sorbent), Waters Chromotography; ODS-4 Octadecyl Silane (500 mg sorbent), Whatman; *or* equivalent)
50- to 100-ml boiling flask
Rotary evaporator with vacuum pump or water aspirator, 40°C
Freeze-resistant container (optional)

1. Condition a C18 cartridge by passing two column volumes methanol through the sorbent bed.
2. Pass three column volumes acidified deionized distilled water through cartridge to remove remaining methanol.
3. Force an aqueous anthocyanin extract through cartridge.

   The solid-phase extractant will become colored. Stop loading sample if excessive color is passing through the cartridge (see Critical Parameters and Troubleshooting, discussion of anthocyanin purification).

   The volume of extract applied to the cartridge will depend mainly on sample quantity, its anthocyanin content, and the amount of sorbent packing in the column. Usually a sample volume of 5 to 10 ml is used for a C18 solid-phase extraction cartridge containing 360 mg sorbent.

4. Wash cartridge with two column volumes acidified water to remove compounds not adsorbed (e.g., sugars, acids).
5. Wash cartridge with two column volumes ethyl acetate to remove polyphenolic compounds such as phenolic acids and flavonols.

   This step is optional, depending on how critical it is to separate anthocyanins from other polyphenolics in subsequent analysis. The ethyl acetate fraction is enriched in polyphenolics such as flavonols, procyanidins, and cinnamates. If analysis of this fraction is desired, a cleaner isolate will be obtained if residual water is removed by passing a nitrogen gas stream through the cartridge for 2 to 3 min before applying ethyl acetate.
6. Elute anthocyanin pigments with acidified methanol and collect in a 50- to 100-ml boiling flask.
7. Remove methanol in a rotary evaporator at 40°C under vacuum.
8. Redissolve pigments in acidified deionized distilled water or an appropriate HPLC mobile-phase solvent.
9. Store purified anthocyanin extract at 4°C if subsequent analysis will be performed within 24 hr. Store sample for longer periods at −15°C or lower (preferably at −70°C) in a freeze-resistant container to minimize pigment degradation.

COMMENTARY

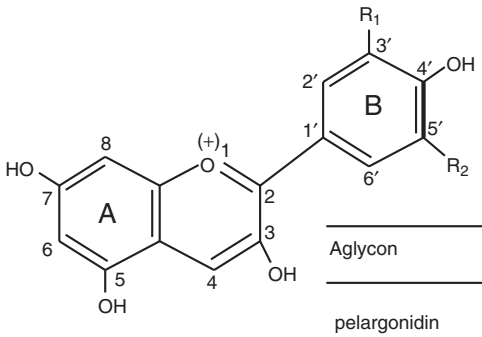
Background Information

Anthocyanins are flavonoid compounds that are widely distributed in plants. They are responsible for blue, purple, violet, magenta, red, and orange plant coloration (Jackman and Smith, 1996). The range of colors associated with anthocyanins results from distinct and varied substitution of the parent $C_6C_3C_6$ (aglycone) nucleus (Figure F1.1.2), in addition to acylation patterns and various environmental influences (Jackman and Smith, 1992).

Extraction

The extraction of anthocyanins is the first step in determination of total as well as individual anthocyanins in any type of plant tissue (Fuleki and Francis, 1968). The choice of an extraction method is of great importance in the analysis of anthocyanins, and largely depends on the purpose of the extraction, the nature of the anthocyanins, and the source material. A good extraction procedure should maximize anthocyanin recovery with a minimal amount of adjuncts and minimal degradation or alteration of the natural (in vivo) state. Nevertheless, one can never be sure that the extracted pigment is exactly the one occurring in vivo (Brouillard and Dangles, 1994). Knowledge of the factors that influence anthocyanin structure and stability is vital and has been reviewed by Markakis (1982), Francis (1989), and Jackman and Smith (1996). It is also desirable that the extraction procedure not be too complex, hazardous, time consuming, or costly.

In most fruits and vegetables the anthocyanin pigments are located in cells near the surface (Jackman et al., 1987). Extraction procedures have generally involved the use of acidic solvents, which denature the membranes of cell tissue and simultaneously dissolve pigments. The acid tends to stabilize anthocyanins, but it may also change the native form of the pigment in the tissue by breaking associations with metals, co-pigments, or other factors. Concentration procedures may also cause acid hydrolysis of labile acyl and sugar residues. Extraction with solvents containing hydrochloric acid may result in pigment degradation during concentration, and is one of the reasons why acylations with aliphatic acids had been overlooked in the past (Strack and Wray, 1989). To minimize the decomposition of pigments, the use of weaker organic acids (e.g., formic, acetic, citric, or tartaric acids) or small amounts (0.5% to 3%) of more volatile acids (e.g., trifluoroacetic acid) that can then be removed during pigment concentration has been proposed (Strack and Wray, 1994; Jackman and Smith, 1996). Alternatives that have been used successfully for extraction include 3% trifluoroacetic acid, 5% acetic acid, and 0.01% to 2.5% citric acid (Metivier et al., 1980; Odake et al., 1992; Baublis et al., 1994). Low hydrochloric acid concentrations (on the order of



Aglycon	Substitution pattern R1	R2	λ_{max} (nm) visible spectra
pelargonidin	H	H	494 (orange)
cyanidin	OH	H	506 (orange-red)
delphinidin	OH	OH	508 (blue-red)
peonidin	OCH_3	H	506 (orange-red)
petunidin	OCH_3	OH	508 (blue-red)
malvidin	OCH_3	OCH_3	510 (blue-red)

Figure F1.1.2 Structural and spectral characteristics of the major naturally occurring aglycons. The chemical structure indicates the two aromatic rings (**A**, **B**) as well as the R_1 and R_2 substitution sites.

0.01% to 0.05%) and procedures in the absence of acid have also been suggested (Jackman et al., 1987; Strack and Wray, 1994). Procedures carried out in the presence of acid need to be performed with care to avoid acid-dependent pigment degradation.

A commonly used method is maceration of crushed or ground material for a few hours to overnight at 4°C in methanol containing small amounts of hydrochloric acid (<1%). The extracted material is usually too dilute for further analyses, and the extraction procedure is usually followed by evaporation of the methanol using vacuum and mild temperatures (30° to 40°C). The extraction of anthocyanins using ethanol acidified with citric acid (0.01%) instead of hydrochloric acid was reported by Main et al. (1978). Ethanol would be preferred for food use to avoid the toxicity of methanolic solutions. Citric acid is less corrosive than hydrochloric acid, chelates metals, maintains a low pH, and may have a protective effect during processing (Timberlake and Bridle, 1980). However, recoveries are not as high as those obtained with methanol, and concentration is more difficult because citric acid has a higher boiling point.

To obtain anthocyanins closer to their natural state, a number of researchers have performed the initial extraction using neutral solvents such as 60% methanol, *n*-butanol, cold acetone, acetone/methanol/water mixtures, or simply water (Jackman et al., 1987). Others have isolated anthocyanin pigments with mixtures of methanol/acetic acid/water (10:1:9, v/v/v; Takeda et al., 1986; Davies and Mazza, 1992), ethanol/acetic acid/water (12:1:24, v/v/v; Toki et al., 1991; 10:1:9, v/v/v; Hosokawa et al., 1995), and methanol/formic acid/water (50:5:45, v/v/v; Donner et al., 1997). Metivier et al. (1980) compared the efficiency of extraction with three different solvents—methanol, ethanol, and water—that were acidified with either hydrochloric acid or different organic acids, and found that methanol extraction was 20% more effective than ethanol and 73% more effective than water when used for anthocyanin recovery from grape pomace. They also reported that hydrochloric acid was most effective when used in combination with ethanol, whereas citric acid was more effective with methanol, and acetic acid was more effective with water.

Depending on the means of extraction, decreasing the ratio of extraction solvent to plant material could avoid the need for a concentration step (Jackman and Smith, 1996).

A second procedure uses acetone as the extracting solvent followed by partition with chloroform (Timberlake and Bridle, 1971; Wrolstad and Heatherbell, 1974; Abers and Wrolstad, 1979; Wrolstad et al., 1990). Timberlake and Bridle (1971) compared this extraction procedure with the usual acidified methanol extraction and concluded that the use of acetone with separation of the aqueous phase by addition of chloroform gave much cleaner and better-defined bands and enabled a better

assessment of anthocyanin composition. Recently, Wrolstad and Durst (1998) compared the acetone/chloroform method of extraction to the acidified methanol method on twenty samples (liquid and dry powder forms) derived from elderberries and cranberries that included juices, colorants, and nutraceutical preparations. Anthocyanin recoveries were comparable or up to 30% higher with the use of acetone. An advantage of this procedure is that the chloroform/acetone mixture will partition lipids, chlorophylls, and other water-insoluble materials from anthocyanins, yielding high recoveries of anthocyanins that require little concentration or further purification. The moisture content of the sample and the water from the secondary acetone extractions determine the final aqueous volume.

Purification of crude extracts

Frequently, the qualitative and quantitative analysis of anthocyanins is complicated by the presence of other compounds that may interfere with the measurements. The solvent systems generally used for extraction purposes are by no means specific for anthocyanins (Markakis, 1974; Jackman et al., 1987). Purification of anthocyanin-containing extracts is often necessary because considerable amounts of other compounds may also be extracted and concentrated. The variety and levels of other compounds will depend on the solvent and methodologies used. The presence of extraneous materials could influence the stability or analysis of anthocyanins. Therefore, the next step towards anthocyanin characterization is the prefractionation of those extracts.

When appreciable amounts of lipids, chlorophylls, or unwanted polyphenols are suspected to be present in anthocyanin-containing extracts, these materials may be removed by washing with petroleum ether, ethyl ether, diethyl ether, or ethyl acetate (Jackman and Smith, 1996).

Different resins have been used to clean up or prefractionate anthocyanins prior to isolation or characterization, including ion-exchange resins, polyamide powders, and gel materials. Chromatography on Dowex or Amberlite ion-exchange resins, as well as polyamide powders such as polyvinylpyrrolidone (PVP), have been used to isolate polar nonphenolic compounds from crude anthocyanin extracts. Column chromatography on Sephadex LH-20 can be used for fractionation of crude extracts and is also particularly useful for purification of individual anthocyanins (Strack and Wray, 1989).

C18 cartridges are becoming popular because of their ease of use and high efficiency for fractionating anthocyanins. In an aqueous phase, anthocyanins are bound to the solid phase, whereas polar compounds such as acids and sugars can be washed away with acidified water. Using ethyl acetate to wash out polyphenolic compounds other than anthocyanins was suggested by Oszmianski and Lee (1990). Finally, a relatively pure anthocyanin extract can be removed from the column with slightly acidified methanol. Baldi et al. (1995) proposed a complex fractionation method for grape (*Vitis vinifera* L.) anthocyanin extracts that involved deposition of the sample on an Extrelut 20 cartridge to remove lipophilic and polyphenolic compounds with hexane and ethyl acetate, followed by adsorption of the sample onto a C18 cartridge to eliminate hydrophilic molecules (e.g., sugars, organic acids).

A key factor in the use of these purification techniques is the stability of anthocyanins under the conditions used as well as the ease of anthocyanin recovery from the column (Strack and Wray, 1989).

Critical Parameters and Troubleshooting

Sample preparation

Grinding the plant material in the presence of liquid nitrogen provides a uniform powdered composite sample; however, some powdered materials are very hygroscopic. With such materials, it is advisable to accurately weigh the plant material (before freezing) and quantitatively transfer the powdered material with an appropriate solvent.

Make sure that the container is completely dried before pouring the liquid nitrogen into the blender because any remaining water will freeze the blender bearings, and the blades will not revolve. If this happens, recover the material and rinse the blender with acetone and air dry. Chill the blender with small amounts of liquid nitrogen, allow to evaporate, and turn the blender on and off before adding frozen plant material.

When working with liquid nitrogen, do not tightly close the lid of the blender or mill container, so that the gas can escape.

Anthocyanin extraction

It is critical that the acetone/chloroform mixture be in an acid environment to avoid

explosion hazards. The use of acidified acetone as extracting solvent will ensure a solution with low pH. In addition, the aqueous fraction will also have a low pH, which favors anthocyanin stability.

The authors have used ratios of acetone/chloroform varying from 1:1, 1:2, 1:2.4, to even 1:5. A greater proportion of chloroform reduces the amount of acetone in the aqueous phase and may eliminate the need for removal of acetone by rotary evaporation. This evaporative step, however, takes little time with no apparent anthocyanin degradation. Therefore, the authors favor using 1:1 or 1:2 ratios to avoid excessive use of the chloroform solvent.

Use a temperature of 30° to 40°C during rotary evaporation to avoid anthocyanin degradation. It is also important to monitor the process to ensure that the aqueous crude extract is not suctioned into the solvent reservoir. If this is going to happen, quickly release the vacuum and transfer the extract to a larger boiling flask and/or reduce the temperature of the water bath.

The crude anthocyanin extract contains a low level of hydrochloric acid (0.01%), which favors the formation of the stable flavylium ion. However, during concentration (and especially at dryness) the acid could cause the hydrolysis of labile acyl groups, co-pigments, or metal complexes that are part of the native form of the anthocyanin and are important for their stability. The risk of concentration of hydrochloric acid in the extract is limited with the acetone/chloroform procedure, as most of the extract contains water (high boiling point) with small levels of organic solvent. However, during methanol (low boiling point) extraction, the alcohol often evaporates to dryness with increased danger of anthocyanin decomposition.

An advantage of the acetone/chloroform method as compared to the methanol method is that the aqueous anthocyanin is not contaminated with lipophilic compounds. If the methanol method is used, the concentrated extract can be extracted with hexane, petroleum ether, or diethyl ether to remove unwanted lipophilic compounds after vacuum evaporation of the methanol (Fuleki and Francis, 1968).

The extracted anthocyanin-containing solution can be used for pigment quantitation by the pH-differential method (*UNIT F1.2*). Both the weight of the fresh material and the final volume of the extract should be accurately recorded when further analyses of pigment content will be carried out.

Anthocyanin purification

Anthocyanin purification steps are important for anthocyanin characterization. Removal of interfering compounds allows for more reliable HPLC separation, spectral information, mass spectra, and NMR spectra during the identification of anthocyanins in plant extracts.

Solid-phase extraction is used to adsorb the anthocyanin and other hydrophobic organic compounds and remove sugars and organic acids from the extract. It is important to avoid saturation of the C18 resin. Polyphenolic compounds along with anthocyanins have an affinity for the C18 column and will compete for binding sites. Use a 10- to 20-g sorbent cartridge placed on a filtration flask for a large extraction volume (50 to 250 ml) and intensely colored extracts (i.e., high levels of anthocyanins). Saturation of the C18 cartridge is clearly evidenced by the leaching of colored compounds (anthocyanins) from the resin. When this occurs, stop adding sample to the cartridge, wash the column with acidified water, and elute the anthocyanins before continuing the purification process.

Ethyl acetate is used on the C18-adsorbed anthocyanins to remove polyphenolic compounds. More efficient polyphenolic recovery will be accomplished if residual water is removed from the cartridge with a nitrogen gas stream for 2 to 3 min before application of ethyl acetate. After washing the column with ethyl acetate, water should not be added because some anthocyanins could be eluted and lost. Ethyl acetate removal of polyphenolics is particularly recommended if the anthocyanin fraction is to be subsequently analyzed by electrospray mass spectroscopy.

Anticipated Results

A homogeneous and fine powder should be obtained from the liquid nitrogen blending process. After extraction and purification of the plant material, a colored solution should be obtained. Depending on the nature of the predominant anthocyanin and its concentration, the coloration of the solution might be pink/red or purple. In the acetone/chloroform procedure, the organic solvent (lower phase) might show coloration due to the presence of chlorophyll (green) and/or carotenoids (yellow, red) in the plant material. The yield will vary depending on the type of materials used.

Time Considerations

The sample preparation should take 15 to 30 min, depending on the amount of material. The

acetone extraction of the anthocyanin pigments usually takes 1 to 2 hr. After addition of chloroform, the solution should be stored overnight at 4°C to obtain well-defined phases. Alternatively, phase separation can be accelerated by centrifugation. This is particularly appropriate when working with small sample sizes. The sample matrix and the cleanness of the phase separation will influence the time for removal of residual solvents (acetone/chloroform) from the aqueous phase. It may be as short as 2 min or extended to 10 to 20 min. Methanol extraction is a faster procedure. Maceration of the nitrogen-powdered material for 1 hr is enough to extract the pigments. Therefore, if there is no need to remove lipophilic compounds from the concentrated extract, a total time of 2 to 4 hr is sufficient to extract the anthocyanins. Purification by solid-phase resin is a fast procedure and normally takes <1 hr.

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Key References

Oszmianski and Lee, 1990. See above.

A description of methods for isolating polyphenolics and anthocyanins from grapes by solid-phase extraction.

Strack and Wray, 1994. See above.

An excellent review of analytical methods for anthocyanin pigment analyses

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Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy

Anthocyanin pigment content has a critical role in the color quality of many fresh and processed fruits and vegetables. Thus, accurate measurement of anthocyanins, along with their degradation indices, is very useful to food technologists and horticulturists in assessing the quality of raw and processed foods. Since many natural food colorants are anthocyanin derived (e.g., grape-skin extract, red-cabbage extract, purple-carrot extract), the same measurements can be used to assess the color quality of these food ingredients. In addition, there is intense interest in the anthocyanin content of foods and nutraceuticals because of possible health benefits such as reduction of coronary heart disease (Bridle and Timberlake, 1996), improved visual acuity (Timberlake and Henry, 1988), antioxidant activities (Takamura and Yamagami, 1994; Wang et al., 1997), and anticancer activities (Karaivanova et al., 1990; Kamei et al., 1995). Substantial quantitative and qualitative information can be obtained from the spectral characteristics of anthocyanins. The protocols described in this unit rely on the structural transformation of the anthocyanin chromophore as a function of pH, which can be measured using optical spectroscopy. The pH-differential method, a rapid and easy procedure for the quantitation of monomeric anthocyanins, is first described (see Basic Protocol 1). In addition, other auxiliary spectrophotometric techniques are used to measure the extent of anthocyanin polymerization and browning (see Basic Protocol 2).

TOTAL MONOMERIC ANTHOCYANIN BY THE pH-DIFFERENTIAL METHOD

BASIC PROTOCOL 1

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra (Fig. F1.2.1). The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5 (Fig. F1.2.2). The pH-differential method is based on this reaction, and permits accurate and rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds.

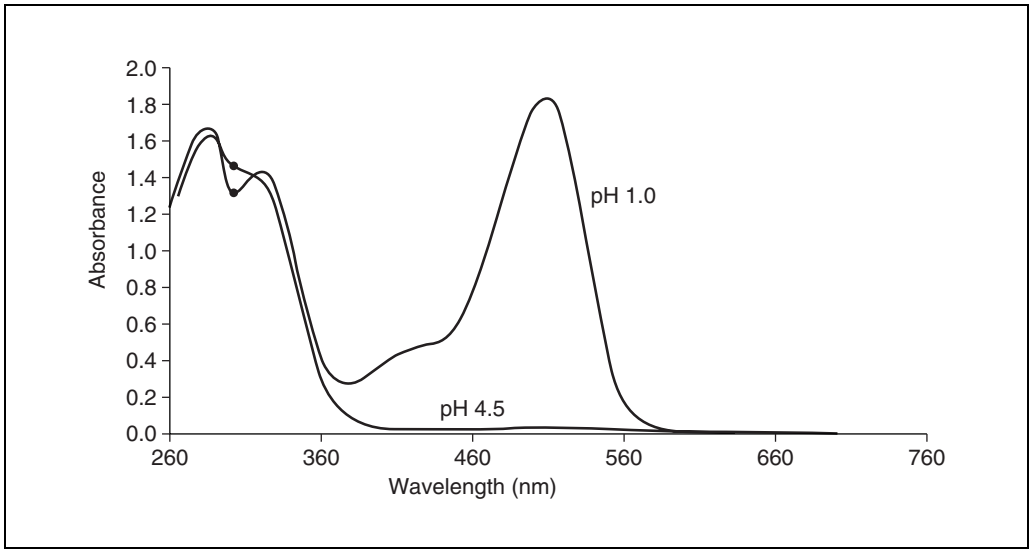

Figure F1.2.1 Spectral characteristics of purified radish anthocyanins (acylated pelargonidin-3-sophoroside-5-glucoside derivatives) in pH 1.0 and pH 4.5 buffers.

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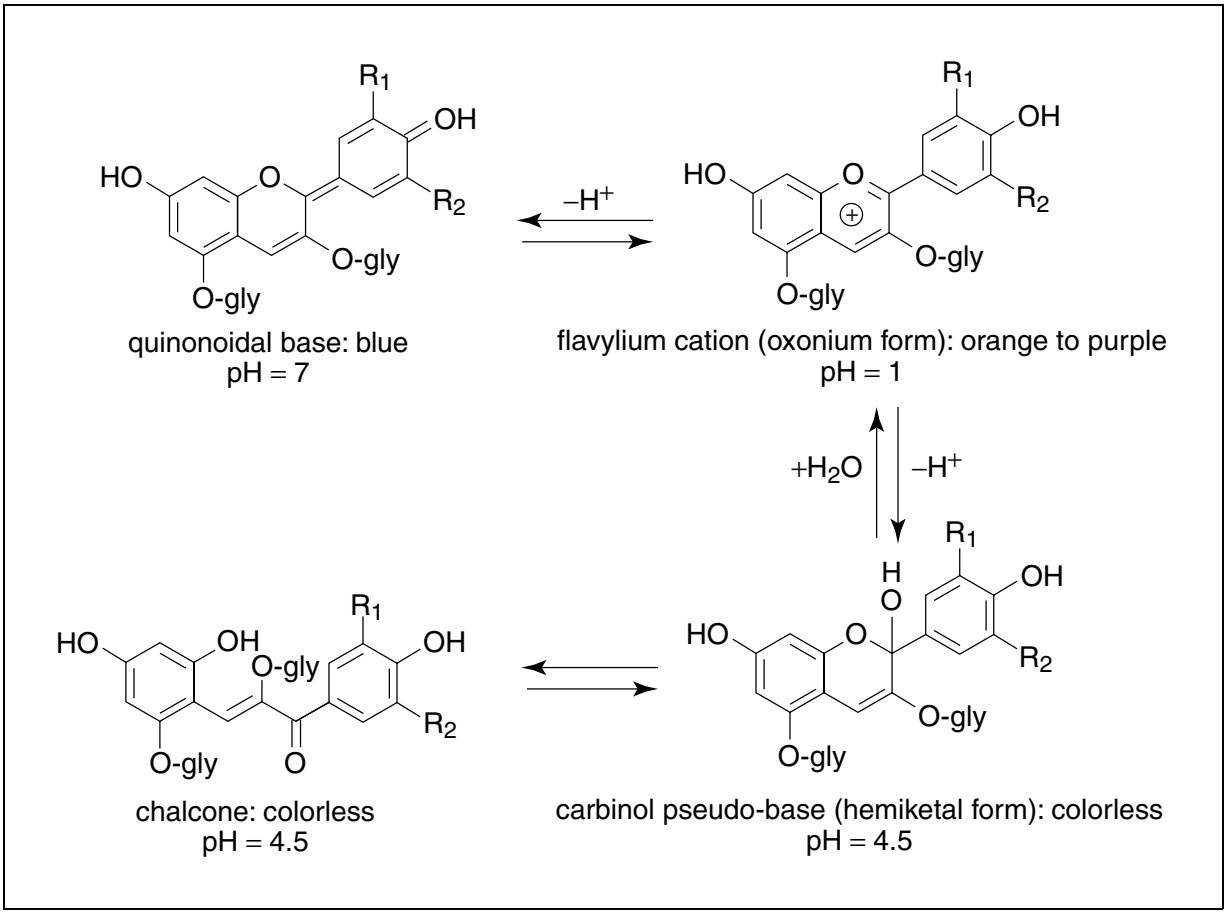

Figure F1.2.2 Predominant structural forms of anthocyanins present at different pH levels.

Materials

0.025 M potassium chloride buffer, pH 1.0 (see recipe)
0.4 M sodium acetate buffer, pH 4.5 (see recipe)

1. Turn on the spectrophotometer. Allow the instrument to warm up at least 30 min before taking measurements.

2. Determine the appropriate dilution factor for the sample by diluting with potassium chloride buffer, pH 1.0, until the absorbance of the sample at the $\lambda_{vis\text{-}max}$ (Table F1.2.1) is within the linear range of the spectrophotometer (i.e., for most spectrophotometers the absorbance should be less than 1.2). Divide the final volume of the sample by the initial volume to obtain the dilution factor (DF; for example see step 7).

   IMPORTANT NOTE*: In order to not exceed the buffer's capacity, the sample should not exceed 20% of the total volume.*

3. Zero the spectrophotometer with distilled water at all wavelengths that will be used ($\lambda_{vis\text{-}max}$ and 700 nm).

   Many spectrophotometers will allow for a rapid baseline correction to zero by using baseline adjust.

4. Prepare two dilutions of the sample, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each by the previously determined dilution factor (step 2). Let these dilutions equilibrate for 15 min.

Table F1.2.1 Reported Molar Absorptivity of Anthocyanins

Anthocyanin[a]	Solvent system	$\lambda_{vis\text{-}max}$ (nm)	Molar absorptivity (ε)	Reference
Cyanidin (Cyd)				
Cyd	0.1% HCl in ethanol	510.5	24600	Schou, 1927
	0.1% HCl in ethanol	547	34700	Ribereau-Gayon, 1959
Cyd-3-ara	15:85 0.1 N HCl/ethanol	538	44400	Zapsalis and Francis, 1965
	15:85 0.1 N HCl/ethanol	535	44460	Fuleki and Francis, 1968a
Cyd-3,5-diglu	0.1 N HCl	520	30175	Niketic-Aleksic and Hrazdina, 1972
	Methanolic HCl	508.5	35000	Brouillard and El Hache Chahine, 1980
Cyd-3-gal	0.1% HCl in methanol	530	34300	Siegelman and Hendricks, 1958
	15:85 0.1 N HCl/ethanol	535	44900	Sakamura and Francis, 1961
	15:85 0.1 N HCl/ethanol	535	46200	Zapsalis and Francis, 1965
	15:85 0.1 N HCl/ethanol	535	46230	Fuleki and Francis, 1968a
	HCl in methanol	530	30200	Swain, 1965
Cyd-3-glu	Aqueous buffer, pH 1	510	26900	Jurd and Asen, 1966
	0.1 N HCl	520	25740	McClure, 1967
	1% HCl in methanol	530	34300	Siegelman and Hendricks, 1958
	10% ethanol, pH 1.5	512	18800	Heredia et al., 1998
Cyd-3-rut	Aqueous buffer, pH 0.9	510	7000	Figueiredo et al., 1996
	1% HCl	523	28840	Swain, 1965
Cyd-3-sam-5-glu	Aqueous buffer, pH 0.9	522	3600	Figueiredo et al., 1996
Cyd-3-sam-5-glu + sinapic + caffeic + malonic	Aqueous buffer, pH 0.9	538	21200	Figueiredo et al., 1996
Cyd-3-sam-5-glu + sinapic + ferulic	Aqueous buffer, pH 0.9	528	15100	Figueiredo et al., 1996
Cyd-3-sam-5-glu + sinapic + ferulic + malonic	Aqueous buffer, pH 0.9	538	20100	Figueiredo et al., 1996
Cyd-3-sam-5-glu + sinapic + p-coum + malonic	Aqueous buffer, pH 0.9	536	19000	Figueiredo et al., 1996
Cyd-3-soph-5-glu	Methanolic HCl	524	37150	Hrazdina et al., 1977
Cyd-3-soph-5-glu + malonic	Methanolic HCl	528	32360	Hrazdina et al., 1977
Cyd-3-soph-5-glu + sinapic	Methanolic HCl	528	37150	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-sinapic	Methanolic HCl	530	38020	Hrazdina et al., 1977
Cyd-3-soph-5-glu + ferulic	Methanolic HCl	528	32360	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-ferulic	Methanolic HCl	530	34670	Hrazdina et al., 1977
Cyd-3-soph-5-glu + p-coumaric	Methanolic HCl	526	38020	Hrazdina et al., 1977
Cyd-3-soph-5-glu + di-p-coumaric	Methanolic HCl	528	32360	Hrazdina et al., 1977
Delphinidin (Dpd)				
Dpd	0.1% HCl in ethanol	522.5	34700	Schou, 1927

continued

Table F1.2.1 Reported Molar Absorptivity of Anthocyanins, continued

Anthocyanin[a]	Solvent system	$\lambda_{vis\text{-}max}$ (nm)	Molar absorptivity (ε)	Reference
Dpd-3-glu	1% HCl in methanol	543	29000	Asen et al., 1959
	10% ethanol, pH 1.5	520	23700	Heredia et al., 1998
Malvidin (Mvd)				
Mvd	0.1% HCl in ethanol	520	37200	Schou, 1927
	0.1% HCl in ethanol	557	36200	Ribereau-Gayon, 1959
Mvd-3,5-diglu	0.1% HCl in ethanol	519	10700	Schou, 1927
	0.1% HCl in ethanol	545	10300	Ribereau-Gayon, 1959
	0.1 N HCl	520	37700	Niketic-Aleksic and Hrazdina, 1972
Mvd-3-glu	0.1% HCl in methanol	546	13900	Somers, 1966
	0.1% HCl in methanol	538	29500	Koeppen and Basson, 1966
	0.1 N HCl	520	28000	Niketic-Aleksic and Hrazdina, 1972
	Methanol, pH 1.0	535	36400	Metivier et al., 1980
	10% ethanol, pH 1.5	520	20200	Heredia et al., 1998
Mvd-3-glu + p-coum	0.1% HCl in methanol	536	30200	Koeppen and Basson, 1966
Pelargonidin (Pg)				
Pg	0.1% HCl in ethanol	504.5	17800	Schou, 1927
	0.025 M potassium chloride buffer, pH 1.0	505	18420	Giusti et al., 1999
	0.1% HCl in methanol	524	19780	Giusti et al., 1999
Pg-3,5-diglu	HCl in methanol	510	32360	Swain, 1965
Pg-3-(dicaffeoylglu)-soph-5-glu	Aqueous buffer, pH 0.8	512	28000	Dangles et al., 1993
Pg-3-glu	1% HCl in H_2O	496	27300	Jorgensen and Geissman, 1955
			36600	Wrolstad et al., 1970
	1% HCl	513	22390	Swain, 1965
	1% HCl in ethanol	516	31620	Swain, 1965
	0.025 M potassium chloride buffer, pH 1.0	496	15600	Giusti et al., 1999
	0.1% HCl in methanol	508	17330	Giusti et al., 1999
Pg-3-rut-5-glu + p-coumaric	0.025 M potassium chloride buffer, pH 1.0	504	32080	Giusti et al., 1999
	0.1% HCl in methanol	511	39591	Giusti et al., 1999
Pg-3-soph-5-glu	Aqueous buffer, pH 0.8	498	18000–20000	Dangles et al., 1993
	0.025 M potassium chloride buffer, pH 1.0	497	25370	Giusti et al., 1999
	0.1% HCl in methanol	506	30690	Giusti et al., 1999
Pg-3-soph-5-glu + ferulic	0.025 M potassium chloride buffer, pH 1.0	506	24140	Giusti et al., 1999
	0.1% HCl in methanol	507	29636	Giusti et al., 1999
Pg-3-soph-5-glu caffeoyl derivatives	Aqueous buffer, pH 0.8	498	18000-20000	Dangles et al., 1993
Pg-3-soph-5-glu + p-coumaric	0.025 M potassium chloride buffer, pH 1.0	506	28720	Giusti et al., 1999
	0.1% HCl in methanol	508	34889	Giusti et al., 1999

continued

Table F1.2.1 Reported Molar Absorptivity of Anthocyanins, continued

Anthocyanin[a]	Solvent system	$\lambda_{vis\text{-}max}$ (nm)	Molar absorptivity (ε)	Reference
Pg-3-soph-5-glu + p-coumaric + malonic	0.025 M potassium chloride buffer, pH 1.0	508	33010	Giusti et al., 1999
	0.1% HCl in methanol	508	39785	Giusti et al., 1999
Pg-3-soph-5-glu + ferulic + malonic	0.025 M potassium chloride buffer, pH 1.0	508	31090	Giusti et al., 1999
	0.1% HCl in methanol	508	39384	Giusti et al., 1999
Peonidin (Pnd)				
Pnd	0.1% HCl in ethanol	511	37200	Schou, 1927
	15:85 0.1 N HCl/ethanol	532	40800	Sakamura and Francis, 1961
Pnd-3-ara	15:85 0.1 N HCl/ethanol	532	46100	Zapsalis and Francis, 1965
	15:85 0.1 N HCl/ethanol	532	46070	Fuleki and Francis, 1968a
Pnd-3,5-diglu	0.1 N HCl	520	36654	Niketic-Aleksic and Hrazdina, 1972
Pnd-3-gal	15:85 0.1 N HCl/ethanol	532	48400	Sakamura and Francis, 1961
	15:85 0.1 N HCl/ethanol	532	48400	Zapsalis and Francis, 1965
	15:85 0.1 N HCl/ethanol	531	48340	Fuleki and Francis, 1968a
Pnd-3-glu	0.1% HCl in methanol	536	11300	Somers, 1966
	10% ethanol, pH 1.5	512	14100	Heredia et al., 1998
Petunidin (Ptd)				
Ptd-3,5-diglu	0.1 N HCl	520	33040	Niketic-Aleksic and Hrazdina, 1972
	HCl in methanol	535	23440	Swain, 1965
Ptd-3-glu	0.1% HCl in methanol	546	12900	Somers, 1966
	10% ethanol, pH 1.5	520	18900	Heredia et al., 1998

[a]Abbreviations: ara: arabinoside; gal: galactoside; glu: glucoside; rut: rutinoside; sam: sambubioside; soph: sophoroside.

5. Measure the absorbance of each dilution at the $\lambda_{vis\text{-}max}$ and at 700 nm (to correct for haze), against a blank cell filled with distilled water.

   All measurements should be made between 15 min and 1 hr after sample preparation, since longer standing times tend to increase observed readings.

   Absorbance readings are made against water blanks, even if the samples are in buffer or bisulfite solutions, as buffer or bisulfite absorbance is nil at the measured wavelengths. The authors have compared the values obtained by using water as a blank as compared with buffer or bisulfite as blanks in different systems and have found no difference in the final values obtained for monomeric and/or polymeric anthocyanin content; on the other hand, reading the diluted samples against the corresponding buffer and/or bisulfite solution is more time-consuming and extends the procedure unnecessarily.

   The samples to be measured should be clear and contain no haze or sediments; however, some colloidal materials may be suspended in the sample, causing scattering of light and a cloudy appearance (haze). This scattering of light needs to be corrected for by reading at a wavelength where no absorbance of the sample occurs, i.e., 700 nm.

Table F1.2.2 Molecular Weights of Anthocyanidins, Anthocyanins, and Acylating Groups Commonly Found in Nature[a]

Anthocyanidins	Pelargonidin	Cyanidin	Peonidin	Delphinidin	Petunidin	Malvidin
	271	287	301	303	317	331
Hex	180.2	180.2	180.2	180.2	180.2	180.2
Hex $-H_2O$[b]	162.2	162.2	162.2	162.2	162.2	162.2
Acd + 1 hex	433.2	449.2	463.2	465.2	479.2	493.2
Acd + 2 hex	595.4	611.4	625.4	627.4	641.4	655.4
Acd + 3 hex	757.6	773.6	787.6	789.6	803.6	817.6
Pent	150.0	150.0	150.0	150.0	150.0	150.0
Pent $-H_2O$[b]	132.0	132.0	132.0	132.0	132.0	132.0
Acd + 1 pent	403.0	419.0	433.0	435.0	449.0	463.0
Acd + 1 hex + 1 pent	565.2	581.2	595.2	597.2	611.2	625.2
Rhamnose	164.2	164.2	164.2	164.2	164.2	164.2
Rutinose	326.2	326.2	326.2	326.2	326.2	326.2
Rutinose $-H_2O$[b]	308.2	308.2	308.2	308.2	308.2	308.2
Acd + rutinose	579.2	595.2	609.2	611.2	625.2	639.2
Acd + rutinose + 1 hex	741.4	757.4	771.4	773.4	787.4	801.4
Acd + rutinose + 1 pent	711.2	727.2	741.2	743.2	757.2	771.2

Common acylating groups

		$-H_2O$[b]
p-Coumaric acid	164.2	146.2
Caffeic acid	180.2	162.2
Ferulic acid	194.2	176.2
Sinapic acid	224	206
Acetic acid	82	64
Propionic acid	96.1	78.1
Malonic acid	104.1	86.1
Succinic acid	118.1	100.1

[a]Abbreviations: hex: hexose; pent: pentose; acd: anthocyanidin.
[b]$-H_2O$ indicates a dehydrated sugar (water is lost upon forming a glycosidic bond).

6. Calculate the absorbance of the diluted sample (A) as follows:

$$A = (A_{\lambda\ \text{vis-max}} - A_{700})_{\text{pH 1.0}} - (A_{\lambda\ \text{vis-max}} - A_{700})_{\text{pH 4.5}}$$

7. Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/liter)} = (A \times \text{MW} \times \text{DF} \times 1000)/(\varepsilon \times 1)$$

where MW is the molecular weight (Table F1.2.2), DF is the dilution factor (for example, if a 0.2 ml sample is diluted to 3 ml, DF = 15), and ε is the molar absorptivity (Table F1.2.1).

IMPORTANT NOTE: *The MW and* ε *used in this formula correspond to the predominant anthocyanin in the sample. Use the* ε *reported in the literature for the anthocyanin pigment in acidic aqueous solvent. If the* ε *of the major pigment is not available, or if the sample composition is unknown, calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 and* ε *= 26,900 (see Background Information, discussion of Molar Absorptivity).*

The equation presented above assumes a pathlength of 1 cm.

INDICES FOR PIGMENT DEGRADATION, POLYMERIC COLOR, AND BROWNING

Indices for anthocyanin degradation of an aqueous extract, juice, or wine can be derived from a few absorbance readings of a sample that has been treated with sodium bisulfite. Anthocyanin pigments will combine with bisulfite to form a colorless sulfonic acid adduct (Figure F1.2.3). Polymerized colored anthocyanin-tannin complexes are resistant to bleaching by bisulfite, whereas the bleaching reaction of monomeric anthocyanins will rapidly go to completion. The absorbance at 420 nm of the bisulfite-treated sample serves as an index for browning. Color density is defined as the sum of absorbances at the $\lambda_{vis\text{-}max}$ and at 420 nm. The ratio between polymerized color and color density is used to determine the percentage of the color that is contributed by polymerized material. The ratio between monomeric and total anthocyanin can be used to determine a degradation index.

Materials

Bisulfite solution (see recipe)
0.025 M potassium chloride buffer, pH 1.0 (see recipe)

1. Turn on the spectrophotometer and allow the instrument to warm up at least 30 min before taking measurements.
2. Determine the appropriate dilution factor for the sample by diluting with 0.025 M potassium chloride buffer, pH 1.0 until the absorbance of the sample at the $\lambda_{vis\text{-}max}$ is within the linear range of the spectrophotometer (i.e., for most spectrophotometers the absorbance should be less than 1.2). Divide the final volume of the sample by the initial volume to obtain the dilution factor (DF; for example see step 6).
3. Zero the spectrophotometer with distilled water at all wavelengths that will be used (420 nm, $\lambda_{vis\text{-}max}$, 700 nm).

   Many spectrophotometers will allow for a rapid baseline correction to zero by using baseline adjust.

4. Dilute the sample with distilled water using the dilution factor already determined (step 2). Transfer 2.8 ml of the diluted sample to each of two cuvettes. Add 0.2 ml of bisulfite solution to one and 0.2 ml distilled water to the other. Equilibrate for 15 min.

   It is critical that the pH not be adjusted to highly acidic conditions (e.g., pH 1) but rather be in the typical pH range of fruit juices and wines, or higher (e.g., pH 3). Highly acidic conditions will reverse the bisulfite addition reaction and render the measurement invalid.

5. Measure the absorbance of both samples at 420 nm, $\lambda_{vis\text{-}max}$, and 700 nm (to correct for haze), against a blank cell filled with distilled water.

   All measurements should be made between 15 min (see step 4) and 1 hr after sample preparation and bisulfite treatment. Longer standing times tend to increase observed readings.

   Absorbance readings are made against water blanks, even if the samples are in buffer or bisulfite solutions, as buffer or bisulfite absorbance is nil at the measured wavelengths. The authors have compared the values obtained by using water as a blank as compared with the use buffer or bisulfite as a blank in different systems and have found no difference in the final values obtained for monomeric and/or polymeric anthocyanin content; on the other hand, reading the samples against the corresponding buffer and/or bisulfite solution is more time-consuming and extends the procedure unnecessarily.

   The samples to be measured should be clear and contain no haze or sediments; however, some colloidal materials may be suspended in the sample, causing scattering of light and a cloudy appearance (haze). This scattering of light needs to be accounted for by reading at a wavelength where no absorbance of the sample occurs (i.e., 700 nm).

6. Calculate the color density of the control sample (treated with water) as follows:

   Color density = $[(A_{420\ nm} - A_{700nm}) + (A_{\lambda\ vis\text{-}max} - A_{700\ nm})] \times DF$

   where DF is the dilution factor (for example, if 0.2 ml sample diluted to 3 ml, DF = 15)

7. Calculate the polymeric color of the bisulfite bleached sample as follows:

   Polymeric color = $[(A_{420\ nm} - A_{700\ nm}) + (A_{\lambda\ vis\text{-}max} - A_{700\ nm})] \times DF$

8. Calculate the percent polymeric color using the formula:

   Percent polymeric color = (polymeric color/color density) × 100

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Bisulfite solution

Dissolve 1 g of potassium metabisulfite ($K_2S_2O_5$) in 5 ml of distilled water.

This reagent must be prepared the same day as the readings; otherwise, it develops a yellow color that will contribute to the absorbance readings and interfere with the quantitation.

Potassium chloride buffer, 0.025 M, pH 1.0

Mix 1.86 g KCl and 980 ml of distilled water in a beaker. Measure the pH and adjust to 1.0 with concentrated HCl. Transfer to a 1 liter volumetric flask and fill to 1 liter with distilled water.

The solution should be stable at room temperature for a few months, but the pH should be checked and adjusted prior to use (see Critical Parameters).

Sodium acetate buffer, 0.4 M, pH 4.5

Mix 54.43 g $CH_3CO_2Na{\cdot}3\ H_2O$ and ~960 ml distilled water in a beaker. Measure the pH and adjust to 4.5 with concentrated HCl. Transfer to a 1 liter volumetric flask and fill to 1 liter with distilled water.

The solution should be stable at room temperature for a few months, but the pH should be checked and adjusted prior to use (see Critical Parameters).

COMMENTARY

Background Information

Anthocyanin pigments are responsible for the attractive red to purple to blue colors of many fruits and vegetables. Anthocyanins are relatively unstable and often undergo degradative reactions during processing and storage. Measurement of total anthocyanin pigment content along with indices for the degradation of these pigments are very useful in assessing the color quality of these foods. Interest in the anthocyanin content of foods and nutraceutical preparations has intensified because of their possible health benefits. They may play a role in reduction of coronary heart disease (Bridle and Timberlake, 1996) and increased visual acuity (Timberlake and Henry, 1988), and also have antioxidant (Takamura and Yamagami, 1994; Wang et al., 1997) and anticancer properties (Karaivanova et al., 1990; Kamei et al., 1995). Anthocyanins have also found considerable potential in the food industry as safe and effective food colorants (Strack and Wray, 1994); interest in this application has increased in recent years. In 1980, the annual world production had been estimated as reaching 10,000 tons from grapes alone (Timberlake, 1980). Quantitative and qualitative anthocyanin composition are important factors in determining the feasibility of the use of new plant materials as anthocyanin-based colorant sources.

Frequently, it is desirable to express anthocyanin determinations in terms that can be compared with the results from different workers. The best way to express these results is in terms

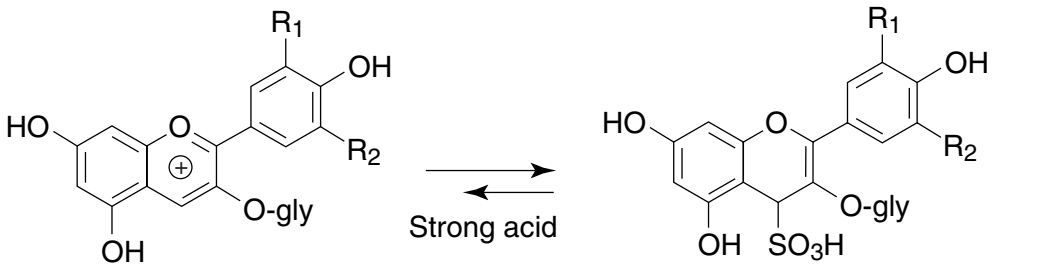

Figure F1.2.3 Formation of colorless anthocyanin-sulfonic acid adducts.

of absolute quantities of anthocyanins present (Fuleki and Francis, 1968a).

The total anthocyanin content in crude extracts containing other phenolic materials has been determined by measuring absorptivity of the solution at a single wavelength. This is possible because anthocyanins have a typical absorption band in the 490 to 550 nm region of the visible spectra (Figure F1.2.1). This band is far from the absorption bands of other phenolics, which have spectral maxima in the UV range (Fuleki and Francis, 1968a). In many instances, however, this simple method is inappropriate because of interference from anthocyanin degradation products or melanoidins from browning reactions (Fuleki and Francis, 1968b). In those cases, the approach has been to use differential and/or subtractive methods to quantify anthocyanins and their degradation products (Jackman and Smith, 1996).

The differential method (see Basic Protocol 1) measures the absorbance at two different pH values, and relies on the structural transformations of the anthocyanin chromophore as a function of pH (Fig. F1.2.1 and Fig. F1.2.2). This concept was first introduced by Sondheimer and Kertesz in 1948, who used pH values of 2.0 and 3.4 for analyses of strawberry jams (Francis, 1989). Since then, the use of other pH values has been proposed. Fuleki and Francis (1968b) used pH 1.0 and 4.5 buffers to measure anthocyanin content in cranberries, and modifications of this technique have been applied to a wide range of commodities (Wrolstad et al., 1982, 1995). The pH differential method has been described as fast and easy for the quantitation of monomeric anthocyanins (Wrolstad et al., 1995).

Subtractive methods (see Basic Protocol 2) are based on the use of bleaching agents that will decolor anthocyanins but not affect interfering materials. A measurement of the absorbance at the visible maximum is obtained, followed by bleaching and remeasuring to give a blank reading (Jackman et al., 1987). The two most used bleaching agents are sodium sulfite (Somers and Evans, 1974; Wrolstad et al., 1982) and hydrogen peroxide (Swain and Hillis, 1959).

By using both of these spectral procedures, accurate measurement of the total monomeric anthocyanin pigment content can be obtained, along with indices for polymeric color, color density, browning, and degradation. To determine total anthocyanin content, the absorbance at pH 1.0 and 4.5 is measured at the $\lambda_{vis\text{-}max}$ (Table F1.2.1) and at 700 nm, which allows for haze correction. The bisulfite bleaching reaction is utilized to generate the various degradation indices. While monomeric anthocyanins are readily bleached by bisulfite at product pH (Fig. F1.2.3), the polymeric anthocyanin-tannin and melanoidin pigments are resistant and will remain colored. Somers and Evans (1974) used this reaction in developing spectral methods for assessing the color quality of wines. The author's laboratory has found them useful for tracking color quality in a wide range of anthocyanin-containing foods (Wrolstad et al., 1982, 1995). Absorbance measurements are taken at the $\lambda_{vis\text{-}max}$ and at 420 nm on the bisulfite bleached and control samples. Color density is the sum of the absorbances at the $\lambda_{vis\text{-}max}$ and at 420 nm of the control sample, while polymeric color is the same measurement for the bisulfite treated sample. A measure of percent polymeric color is obtained as the ratio between these two indexes. The absorbance at 420 nm of the bisulfite-treated sample is an index for browning, as the accumulation of brownish

degradation products increases the absorption in the 400 to 440 nm range. The absorption of these compounds are in general not affected by the addition of a bisulfite solution.

Molar absorptivity

Regardless of the method used for anthocyanin quantitation, the determination of the amount present requires an absorptivity coefficient. Absorptivity coefficients have been reported as the absorption of a 1% solution measured through a 1-cm path at the $\lambda_{vis\text{-}max}$, or as a molar absorption coefficient. Absorptivity coefficients of some known anthocyanins have been reported by different researchers (Table F1.2.1). Through the years, there has been a lack of uniformity on the values of absorptivity reported, mainly due to the difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions (Fuleki and Francis, 1968a; Francis, 1982; Giusti et al., 1999). Other problems are that the anthocyanin mixtures may be very complicated, and not all absorptivity coefficients may be known. Even when they are known, it is necessary to first evaluate if the objective is the estimation of total anthocyanin content or the determination of individual pigments, and then to decide which absorption coefficient(s) to use. The absorptivity is dependent not only on the chemical structure of the pigment but also on the solvent used; preferably, the coefficient used should be one obtained in the same solvent system as the one used in the experiment. If the identity of the pigments is unknown, it has been suggested that it can be expressed as cyanidin-3-glucoside, since that is the most abundant anthocyanin in nature (Francis, 1989).

Spectral characteristics

Substantial information can be obtained from the spectral characteristics of anthocyanins (Fig. F1.2.1). Two distinctive bands of absorption, one in the UV-region (260 to 280 nm) and another in the visible region (490 to 550 nm) are shown by all anthocyanins. The different aglycons have different $\lambda_{vis\text{-}max}$, ranging from 520 nm for pelargonidin to 546 nm for delphinidin, and their monoglucosides exhibit their $\lambda_{vis\text{-}max}$ at about 10 to 15 nm lower (Strack and Wray, 1989). The shape of the spectrum may give information regarding the number and position of glycosidic substitutions and number of cinnamic acid acylations. The ratio between the absorbance at 440 nm and the absorbance at the $\lambda_{vis\text{-}max}$ is almost twice as much for anthocyanins with glycosidic substitutions in position 3 as compared to those with substitutions in positions 3 and 5 or position 5 only. The presence of glycosidic substitutions at other positions (e.g., 3,7-diglycosides) can be recognized because they exhibit a different spectral curve from those of anthocyanins with common substitution patterns. The presence of cinnamic acid acylation is revealed by the presence of a third absorption band in the 310 to 360 nm range (Figure F1.2.1), and the ratio of absorbance at 310 to 360 nm to the absorbance at the visible $\lambda_{vis\text{-}max}$ will give an estimation of the number of acylating groups (Harborne, 1967; Hong and Wrolstad, 1990). The solvent used for spectral determination will affect the position of the absorption bands, and therefore must be taken into consideration when comparing available data.

Critical Parameters and Troubleshooting

The pH of buffers should always be checked and adjusted prior to use. The use of buffers with lower or higher pH levels will result in under- or overestimations of the pigment content.

The accuracy of the results will be greatly affected by the accuracy of the volumetric measurements. Make sure that any volumetric flasks or pipets used for obtaining the appropriate dilutions are calibrated correctly.

For the methodologies described in this unit, all spectral measurements should be made between 15 min and 1 hr after the dilutions have been prepared. The observed readings tend to increase with time.

When working with several different samples, it may be acceptable to use one common approximate $\lambda_{vis\text{-}max}$ that is typical of all samples (i.e., 520 nm). The visible absorbance peak is broad, and measuring a few nanometers off $\lambda_{vis\text{-}max}$ will not significantly alter the estimated final values.

Serial dilutions are recommended to ensure accurate measurements of highly concentrated, high density, or dried samples. Perform a weight-by-volume dilution with distilled water to obtain a single-strength solution (e.g., usually around 10° Brix for fruit juices; *UNIT H1.4*), followed by a second dilution using 0.025 M potassium chloride buffer, pH 1.0. Both dilution factors must be considered when calculating monomeric anthocyanin content.

For example, 1 g of a 75° Brix juice concentrate was diluted to a final volume of 10 ml with distilled water (dilution factor = 10; assuming

Table F1.2.3 Anthocyanin Content of Some Common Fruits and Vegetables

Source	Pigment content (mg/100 g fresh weight)	Reference
Apples (Scugog)	10	Mazza and Miniati, 1993
Bilberries	300–320	Mazza and Miniati, 1993
Blackberries	83–326	Mazza and Miniati, 1993
Black currants	130–400	Timberlake, 1988
Blueberries	25–495	Mazza and Miniati, 1993
Red cabbage	25	Timberlake, 1988
Black chokeberries	560	Kraemer-Schafhalter et al., 1996
Cherries	4–450	Kraemer-Schafhalter et al., 1996
Cranberries	60–200	Timberlake, 1988
Elderberry	450	Kraemer-Schafhalter et al., 1996
Grapes	6–600	Mazza and Miniati, 1993
Kiwi	100	Kraemer-Schafhalter et al., 1996
Red onions	7–21	Mazza and Miniati, 1993
Plum	2–25	Timberlake, 1988
Red radishes	11–60	Giusti et al., 1988
Black raspberries	300–400	Timberlake, 1988
Red Raspberries	20–60	Mazza and Miniati, 1993
Strawberries	15–35	Timberlake, 1988
Tradescantia pallida (leaves)	120	Shi et al., 1992

a density of 1 g/ml for juice). Then, the appropriate dilution factor for the sample was determined by diluting 0.2 ml of the solution with 2.8 ml of 0.025 M potassium chloride buffer, pH 1.0 (dilution factor = 15). To calculate monomeric anthocyanin content, color density, or polymeric color, the dilution factor to use would be: DF = (10 × 15) = 150.

The methodologies used to measure color density and polymeric color were developed for fruit juices, which naturally have an acidic pH. If the material to be measured has a pH in the neutral or alkaline range, the pH of the solution should be lowered with a weak acid. In these cases, the authors recommend the use of a 0.1 M citric acid buffer, pH 3.5, instead of distilled water to prepare the different dilutions.

Some potential interfering materials are other red pigments: FD&C Red No. 40, FD&C Red No. 3, cochineal, and beet powder (betalain pigments). The presence of alternative colorants may be suspected if the $\lambda_{vis\text{-}max}$ at pH 1.0 is high (550 nm, more typical of betalain pigments), or if a bright red coloration is found at pH 4.5 (potential presence of artificial dyes).

The presence of ethanol does not interfere with the assay at the levels typically encountered in wines (10% to 14%).

Highly acylated anthocyanins may not respond to pH changes the same way as anthocyanins with no or few acylating groups, and may not decolor as much as nonacylated or mono- or diacylated anthocyanins do at pH 4.5.

Anticipated Results

The anthocyanin content of different common fruits and vegetables is presented in Table F1.2.3. Anthocyanin-containing fruit or vegetable juices typically have pigment content ranging from 50 to 500 mg/liter. Anthocyanin-based natural colorants and nutraceuticals may have a much higher pigment concentration, on the order of a few grams/liter.

Fresh fruit or vegetable juices should have a low percentage of polymeric color (usually less than 10%), while processed samples and materials subjected to storage abuse will be much higher (30% or more). This is highly variable, dependent on the commodity, processing conditions, and storage history.

Always express anthocyanin pigment content in terms of the specific anthocyanin used for calculation, and specify molecular weight and ε utilized.

Time Considerations

Quantitation of anthocyanins can be achieved in <1 hr. It is necessary to wait for the spectrophotometer to warm up, and for the diluted samples to equilibrate at least 15 min. The absorbance readings take a few minutes.

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Key References

Giusti et al.,1999. See above.

Compares the molar absorptivity of many anthocyanins in different solvent systems.

Somers and Evans, 1974. See above.

Spectral methods are described for generating several color quality indices for wines.

Wrolstad et al., 1982. See above.

Description of the pH differential method for determination of total anthocyanins and indices for anthocyanin degradation as applied to fruit juices and wines.

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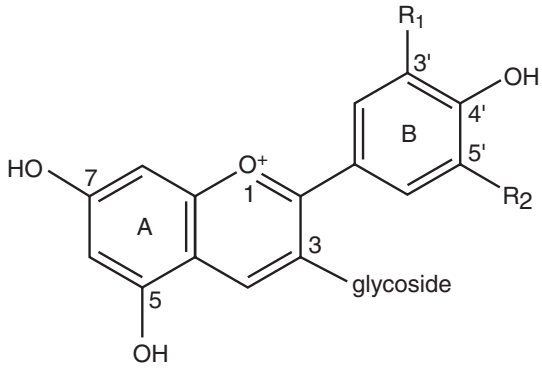
Separation and Characterization of Anthocyanins by HPLC

Anthocyanins are water-soluble pigments which impart the red, purple, and blue coloration of many fruits, vegetables, and cereal grains. Their analysis is useful for commodity identification since the anthocyanin fingerprint pattern is distinctive for different commodities. The authors' laboratory has demonstrated how HPLC anthocyanin analyses can be effectively applied to determine the authenticity of various anthocyanin containing fruit juices (Wrolstad et al., 1994).

Two primary analytical methods for HPLC separation of anthocyanins are described (see Basic Protocol 1 and Alternate Protocol). Basic Protocol 1 has a shorter analysis time and is appropriate for matrices containing simpler anthocyanin glycosides, while the Alternate Protocol is longer and more suitable for those with more complex, nonpolar, acylated anthocyanins. In addition, a couple of auxiliary procedures are presented that are useful for characterization of unknown anthocyanin peaks. The first of these simplifies a chromatogram through acid hydrolysis, thus removing the sugar group(s) and any attached acyl groups from the anthocyanin to form the anthocyanidin (aglycon; see Basic Protocol 2). There are only six anthocyanidins that occur in nature (Figure F1.3.1), so a chromatogram can often be greatly simplified by this treatment. It also helps to confirm the identity of the parent compound or compounds by identification of the aglycon. The final technique discussed is base saponification to remove acylating groups that may be attached to some of the anthocyanins (see Basic Protocol 3). This procedure can often be used prior to and in conjunction with acid hydrolysis to more fully characterize a particular compound. Identification of the sugars can be accomplished by GLC of their tri-methylsilyl derivatives and acylating acids by HPLC (Gao and Mazza, 1994). For more complete identification, additional techniques (e.g., electrospray mass spectroscopy and NMR; Giusti et al., 1999) are necessary for determining the molecular weight, the nature of glycosidic linkages, and the sites of acyl and sugar substitution.

The protocols presented here allow one to analyze the anthocyanins in fruit juices, natural colorants, and extracts from various anthocyanin sources. These profiles are useful for the identification of species, varieties, and for quality assessment of commercial products. They are also used to detect misbranding or adulteration of fruit products with other anthocyanin containing fruits, juices, or colorants.



R_1=R_2=H	Pelargonidin
R_1=OH, R_2=H	Cyanidin
R_1=OCH_3, R_2=H	Peonidin
R_1=R_2=OH	Delphinidin
R_1=OCH_3, R_2=OH	Petunidin
R_1=R_2=OCH_3	Malvidin

Figure F1.3.1 Generalized structure for anthocyanin pigments. A and B rings are labelled.

Contributed by Robert W. Durst and Ronald E. Wrolstad

NOTE: While a variable wavelength UV-Vis detector is sufficient for the protocols in this unit, a UV-Vis diode array detector, which collects spectra for individual peaks, greatly enhances the methods.

BASIC PROTOCOL 1

SAMPLE PREPARATION OF ANTHOCYANINS AND THEIR HPLC SEPARATION ON SILICA C_{18} COLUMNS

This method is used for anthocyanin samples of lesser complexity that do not contain acylated pigments. It is the most common method used for anthocyanin analysis and is effective for most commodities; the major exceptions being red grape and cabbage containing products. This protocol describes the dilution, filtration, and reversed-phase HPLC analysis of these samples.

Materials

Acidified H_2O: add 2 to 4 drops of HCl (~0.01% final) in 500 ml H_2O in a wash bottle

4% phosphoric acid (H_3PO_4)

Acetonitrile

10% acetic acid/5% acetonitrile/1% phosphoric acid in water, filter sterilized: store up to 1 month at 25°C

Anthocyanin standards (optional): cranberry juice cocktail or purified anthocyanins (Extrasynthese or Polyphenols AS)

0.45-µm syringe filters suitable for aqueous samples

HPLC system capable of generating binary gradients, detector capable of monitoring at 520 nm, and reversed-phase C_{18} column (e.g., Phenomenex Prodigy ODS-3 or Supelco LC-18)

1. Dilute samples with water, acidified water, or 4% phosphoric acid, as appropriate to confine the absorbance of the peaks within the limits of the detector as determined by trials. Filter through 0.45-µm filter.

   For most applications, this is all the sample preparation that is necessary. Appropriate dilution is deceptive as it will vary from one commodity to another dependent on the number of anthocyanins in the matrix. The visual appearance and absorptivity of a sample is often not a sufficient indicator for HPLC peak height/area, as samples that have many anthocyanin peaks will need to be more concentrated than a sample that has the same total anthocyanin content but only a single major peak. Most anthocyanin-containing fruit juices will give a suitable response between single strength juice and a dilution of 1:5.

2. Set the flow rate of the HPLC system to 1 ml/min across a 5-µm × 250-mm × 4.6-mm Prodigy ODS-3 column (or equivalent) at ambient temperature. Set the detector at 520 nm. Inject 50 µl sample into the HPLC system and start a gradient similar to that

Table F1.3.1 HPLC Gradient for Anthocyanin Separation on Silica C_{18} Columns

Time (min)	Percent A[a]	Percent B[b]
0	0	100
5	0	100
20	20	80
25[c]	40	60
30	0	100

[a]Solvent A is acetonitrile (CH_3CN).

[b]Solvent B is 10% acetic acid/5% acetonitrile/1% phosphoric acid in water.

[c]Useful data ends at 25 min.

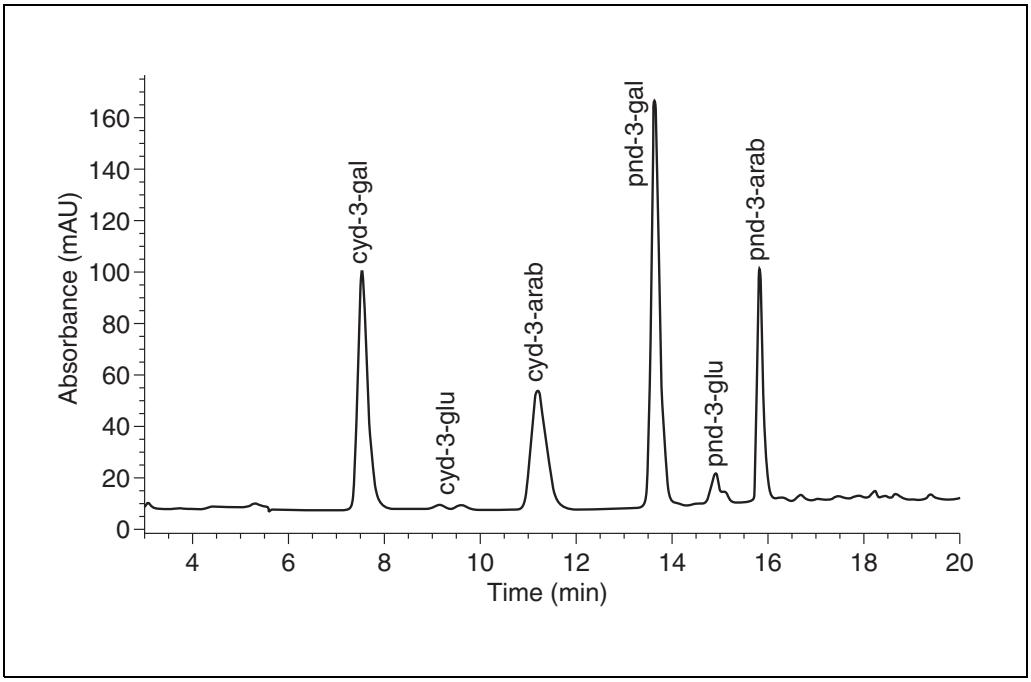


Figure F1.3.2 HPLC chromatogram of cranberry juice. Peaks identified on figure.

outlined in Table F1.3.1. Analyze data as described (see Data Analysis in Critical Parameters and Troubleshooting).

The exact gradient will have to be determined by the analyst, as variations in column brands causes significant differences in the retention times of the peaks of interest. A good material to use as a reference sample is commercial cranberry juice cocktail. It has a simple profile of four major peaks and two minor ones that cover the retention times of most peaks of interest, and will give the experimenter some assurance that the analytical system is behaving satisfactorily. See Figure F1.3.2 for a sample chromatogram with the peaks identified. Another alternative is purified anthocyanins.

Suggested changes are to increase the percentage A at the beginning if the retention time for early peaks are considerably longer than that shown in Figure F1.3.2 or decrease it at 20 min if the later peaks are not well resolved (see Critical Parameters and Troubleshooting).

The step from 20 to 25 min is included to wash highly-retained, non-polar materials from the column. If one sees significant anthocyanin peaks in this region, it may indicate that the sample contains acylated anthocyanins, and that the Alternate Protocol would be more appropriate for that particular sample.

SAMPLE PREPARATION OF ACYLATED ANTHOCYANINS AND THEIR SEPARATION ON POLYMERIC C_{18} COLUMNS

ALTERNATE PROTOCOL

This method is used for complex anthocyanin matrices which usually contain acylated anthocyanins. It can be used for nonacylated pigments also, but because of the extra effort involved in sample preparation and the longer run times involved, it is generally not used for these compounds. Commodities appropriate for this protocol include red grapes, red cabbage, and blueberries. This protocol describes the dilution, sample preparation (including solid phase extraction), filtration, and reversed-phase HPLC analysis of samples using a polymeric based C_{18} column. Steps 1 to 4 are used to separate the anthocyanins from sugars, acids, and other water soluble compounds that are likely present in the sample.

Additional Materials *(also see Basic Protocol 1)*

Acidified methanol: add 2 to 4 drops of HCl (~0.01% final) to 500 ml methanol in a wash bottle

C_{18} solid phase extraction (SPE) cartridges (e.g., C_{18} Sep-Pak; Waters Chromatography)

Syringe

Rotary evaporator and appropriate flasks

5-µm × 250-mm × 4.6-mm polymeric support reversed phase (C_{18}) column (e.g., Polymer Labs PLRP-S)

Isolate anthocyanins

1. Activate a C_{18} SPE cartridge through successive applications of 5 ml acidified methanol and 5 ml acidified water.

2. Using a syringe, apply an anthocyanin containing sample (typically 1 ml) to an activated C_{18} SPE cartridge. Wash twice with 5 ml water each time to remove sugars and acids.

   If this is a qualitative analysis, quantities are not critical, apply as much material as can be conveniently loaded on the SPE cartridge without excessive bleed. If one is performing quantitative analysis, then careful measurements of the volume applied to the SPE cartridge, and the final volume after evaporation and after solvation are necessary.

3. Elute the anthocyanins with two 5-ml aliquots of acidified methanol and collect eluate in a flask that can be put on a rotary evaporator.

4. Remove the acidified methanol from the sample on a rotary evaporator until a small drop of liquid is still present.

   Try not to evaporate the sample all the way to dryness as it will be difficult to dissolve.

   This constitutes the purified anthocyanin fraction that is subjected to further sample preparation in this protocol and elsewhere (see Basic Protocols 2 and 3).

Prepare sample

5. Dissolve the sample in 4% phosphoric acid, dilute as necessary, and pass through a 0.45-µm filter.

   Appropriate dilution is deceptive as it will vary from one commodity to another dependent on the number of anthocyanins in the matrix. The visual appearance and absorptivity of a sample is often not a sufficient indicator for HPLC peak height/area, as samples that have many anthocyanin peaks will need to be more concentrated than a sample that has the same total anthocyanin content but only a single major peak.

   Most anthocyanin-containing fruit juices will give a suitable response between single strength juice and a dilution of 1:5.

Table F1.3.2 HPLC Gradient for Anthocyanin Separation on Polymeric C_{18} Columns

Time	Percent A[a]	Percent B[b]
0	6	94
55	25	75
65[c]	25	75
70	6	94

[a]Solvent A is acetonitrile (CH_3CN).

[b]Solvent B is 4% phosphoric acid (H_3PO_4) in water.

[c]Useful data ends at 65 min.

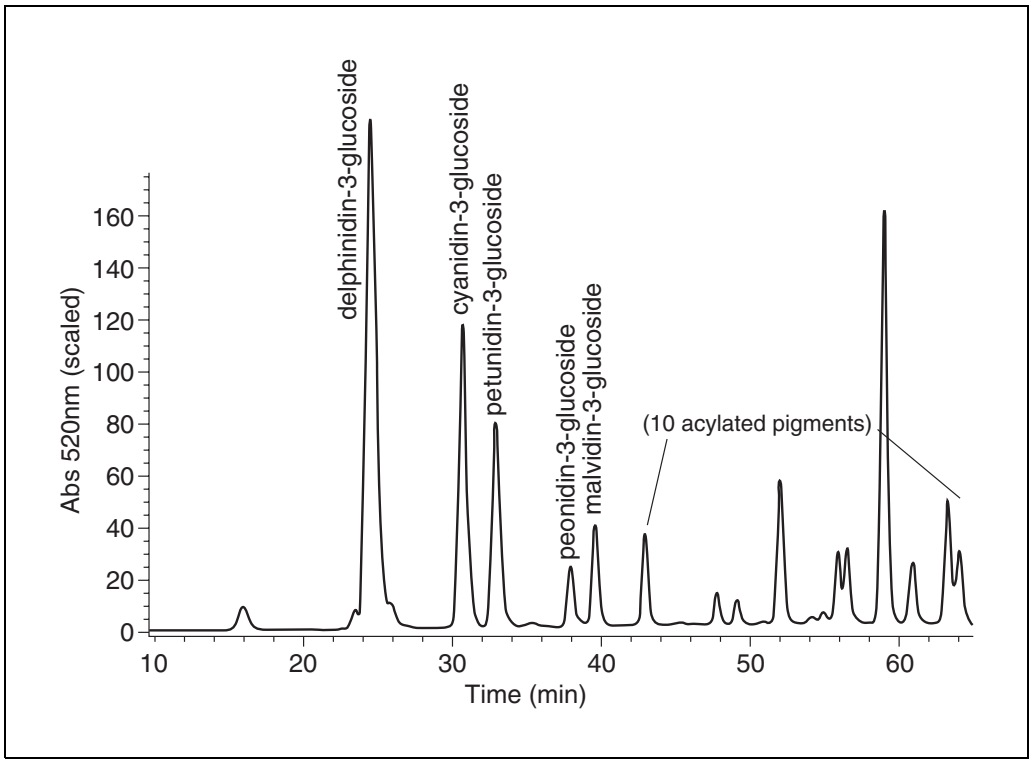


Figure F1.3.3 HPLC chromatogram of concord grape juice. Peaks identified on figure.

Analyze

6. Set the flow rate of the HPLC system to 1 ml/min across a 5-µm × 250-mm × 4.6-mm C_{18} polymeric support reversed-phase column or equivalent at ambient temperature. Set the detector at 520 nm. Inject 50 µl sample into the HPLC system and start a gradient similar to that outlined in Table F1.3.2. Analyze data as described (see Data Anaylsis in Critical Parameters and Troubleshooting).

   The exact gradient will need to be determined by the analyst, as variations in column brands cause significant differences in the retention times of the peaks of interest. A good sample to use as a working standard is concord grape juice. It has a complicated profile of 7 major peaks and up to 10 minor ones that cover the range of retention times for most peaks of interest, and will give the experimenter some assurance that the system is behaving satisfactorily. See Figure F1.3.3 for a sample chromatogram with peak identification.

   Suggested changes are to increase the percentage of A at the beginning if the retention time for early peaks are considerably longer than that shown in Figure F1.3.3, or decrease it at 55 min if the later peaks are not well resolved (see Critical Parameters and Troubleshooting).

BASIC PROTOCOL 2

ANTHOCYANIDINS: PREPARATION AND HPLC

This method is used to simplify a chromatogram by reducing the number of compounds in a sample to the six aglycons. This protocol describes the dilution, preparation (including solid phase extraction and acid hydrolysis), filtration, and reversed-phase HPLC analysis of the sample.

Materials

Acidified methanol and H_2O: add 2 to 4 drops of HCl (~0.01% final) to 500 ml methanol or H_2O in a wash bottle
2 N HCl
Nitrogen gas
4% phosphoric acid
Acetonitrile
10% acetic acid/5% acetonitrile/1% phosphoric acid in water, filter sterilized: store up to 1 month at 25°C
Anthocyanidin standards (optional): e.g., concord grape juice (cyanidin, delphinidin, malvidin, peonidin, and petunidin), strawberry juice (cyanidin and pelargonidin)

C_{18} solid phase extraction (SPE) cartridges (C_{18} Sep-Pak; Waters Chromatography)
Rotary evaporator and appropriate flasks
20-ml screw-top test tubes with Teflon-lined caps
Boiling water and ice baths
0.45-µm filters
HPLC system (see Basic Protocol 1)

1. Isolate anthocyanin fraction (see Alternate Protocol, steps 1 to 4).
2. Dissolve the sample in 10 ml 2 N HCl and transfer to a 20-ml screw-top test tube. Flush the tube with nitrogen gas and seal with Teflon-lined cap.
3. Place tube in a boiling water bath and allow to hydrolyze for 30 min.
4. Cool sample in an ice bath.
5. Apply sample to an activated C_{18} SPE cartridge and rinse twice with 5 ml water each time. Elute anthocyanins twice with 5 ml acidified methanol each time and collect in a flask appropriate for a rotary evaporator.
6. Remove acidified methanol from the sample on the rotary evaporator until a small drop of liquid is still present.

   Try not to evaporate the sample all the way to dryness. It makes redissolving difficult.

7. Dissolve the sample in 2 to 5 ml 4% phosphoric acid, filter through 0.45-µm filter and place in a 20-ml screw-top test tube. Flush the tube with nitrogen, seal with a Teflon-lined cap, and store on ice in the dark until injection.

   The anthocyanidins are unstable and very prone to oxidation and light induced polymerization, hence these precautions. The sample should be analyzed within 60 min or significant degradation may occur in spite of the above precautions.

8. Set the flow rate of the HPLC system to 1 ml/min across a 5-µm × 250-mm × 4.6-mm Prodigy ODS-3 column (or equivalent) at ambient temperature. Set the detector at 520 nm. Inject 50 µl sample into the HPLC system and start a gradient similar to that outlined in Table F1.3.3. Analyze data as described (see Data Analysis in Critical Parameters and Troubleshooting).

The exact gradient will have to be determined by the analyst. Variations in column brands show significant differences in the retention times of the peaks of interest. A good sample to use as a working standard is commercial concord grape juice. It contains five of the six anthocyanidins (it is missing pelargonidin). Pelargonidin can be prepared by hydrolyzing a strawberry sample, which should contain pelargonidin (~90%) and small amounts of cyanidin. See Figure F1.3.4 for a sample chromatogram with the peaks identified.

Suggested changes would be to increase the percentage of solution A at the beginning if retention times are excessively long or decrease it at 20 min if there is incomplete resolution. It is not unusual to have minor amounts of unhydrolyzed anthocyanins glycosides in the sample. If amounts are excessive, increase the hydrolysis time or decrease the amount of sample subjected to hydrolysis.

Table F1.3.3 HPLC Gradient for Anthocyanidin Separation on C_{18} Silica Columns

Time	Percent A[a]	Percent B[b]
0	5	95
20[c]	20	80
25	5	95

[a]Solvent A is acetonitrile (CH_3CN).
[b]Solvent B is 10% acetic acid/5% acetonitrile/1% phosphoric acid in water.
[c]Useful data ends at 20 min.

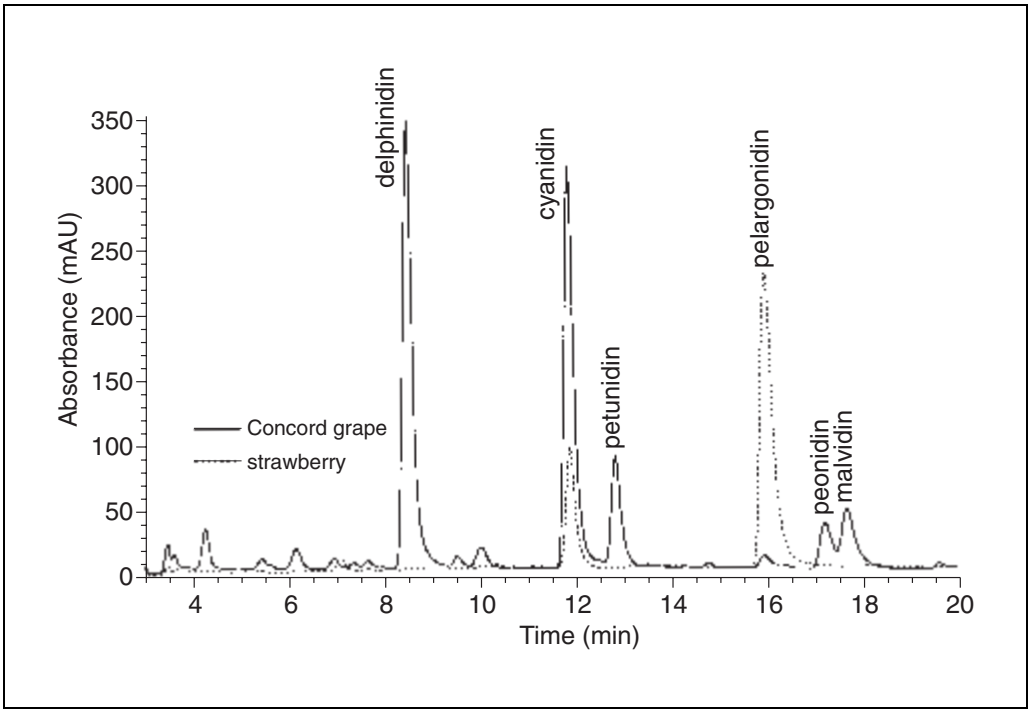


Figure F1.3.4 HPLC chromatogram of concord grape (solid line) and strawberry (dashed line) anthocyanins. Peaks identified on figure.

BASIC PROTOCOL 3

SAPONIFICATION OF ACYLATED ANTHOCYANINS AND THEIR HPLC SEPARATION

This method clearly shows which anthocyanin peaks in a sample matrix contain acyl substituents. This protocol describes sample dilution and preparation (including solid phase extraction and saponification). Subsequent HPLC analysis will be via one of the protocols already described for anthocyanin separation (see Basic Protocol 1 and Alternate Protocol).

Materials

Acidified methanol and H_2O: add 2 to 4 drops of HCl (~0.01% final) to 500 ml methanol or H_2O in a wash bottle
10% (w/v) KOH
2 N HCl
4% phosphoric acid
Nitrogen gas (optional)

C_{18} solid phase extraction (SPE) cartridges (e.g., C_{18} Sep-Pak; Waters Chromatography)
Rotary evaporator and appropriate flasks
20-ml screw-top test tubes with Teflon-lined caps
0.45-µm filters

1. Isolate anthocyanin fraction (see Alternate Protocol, steps 1 to 4).
2. Dissolve the sample in ~10 ml 10% (w/v) KOH in a 20-ml screw-top test tube with Teflon-lined cap and allow to react at room temperature for 8 to 10 min in the dark. Neutralize the sample with ~5 ml 2 N HCl.
3. Apply sample to an activated C_{18} SPE cartridge. Rinse twice with 5 ml water each time. Elute anthocyanins by adding 5 ml acidified methanol twice and collect in a rotary evaporator flask.
4. Remove acidified methanol from the sample on the rotary evaporator until a small drop of liquid is still present.

   Try not to evaporate the sample all the way to dryness. It makes redissolving difficult and may also induce hydrolysis of the glycosidic substituents.

5. Dissolve the sample in 2 to 5 ml 4% phosphoric acid, filter through a 0.45-µm filter and place in a 20-ml screw-top test tube with Teflon-lined cap.

   The anthocyanins are generally stable, but should be analyzed within a day or so. For samples that need to be stored longer, flush tube with nitrogen and store refrigerated or frozen.

6. Analyze the sample by an appropriate HPLC procedure (see Basic Protocol 1 or Alternate Protocol).

   The choice of which protocol to use is determined by data interpretation needs. Basic Protocol 1 may be appropriate since the sample should no longer contain any acylated anthocyanins. If one needs to compare the saponified chromatogram with that of the unsaponified sample to determine which peaks are acylated, then using the Alternate Protocol would be the better choice since direct comparisons of the two can then be made. One should see the decrease (or complete loss) of acylated peaks and the appearance of or increase in unacylated anthocyanin peaks liberated by saponification.

COMMENTARY

Background Information

Anthocyanins are the water-soluble pigments which impart the red, purple, and blue coloration of many fruits, vegetables, and cereal grains. These pigments are largely responsible for the color characteristics of raw and processed products. Their analysis is useful for commodity identification since the anthocyanin fingerprint pattern is distinctive for different foods. Figure 1.3.1 shows the generalized structure for anthocyanin pigments. There are six common anthocyanidins (aglycons liberated from anthocyanins by acid hydrolysis). Structural variations are greatly expanded through varying patterns of glycosidic substitution at the 3 and 5 positions. Additional variations can occur through acylation of the sugar substituents with organic acids. Over 300 different anthocyanin pigments have been found in nature (Strack and Wray, 1994). The anthocyanin pigment patterns exhibited by different species and even different varieties of the same species have proven useful in chemotaxonomic investigations of higher plants (Harborne and Turner, 1984). The authors have demonstrated how HPLC anthocyanin analyses can be effectively applied to determine the authenticity of various anthocyanin containing fruit juices (Wrolstad et al., 1994).

HPLC has become the method of choice for analysis of anthocyanin pigments. It offers several advantages over the traditional techniques of paper or thin-layer chromatography (TLC) such as greater resolution, shorter analysis times, and easy quantitation. Reversed-phase chromatography also has the attractive feature of predictability of elution order based on polarity; triglycosides typically elute before diglycosides, which elute before monoglycosides. An exception to this generalization are the -3-rutinosides which have longer retention times than the corresponding -3-glucosides because of the nonpolarity imparted by the C-6 methyl group of rhamnose. Glycosides of hexoses are more polar and elute earlier than glycosides of pentose sugars, e.g., -3-galactosides elute prior to -3-arabinosides. The elution order of the aglycons can be predicted on the basis of the number of hydrophilic phenolic and hydrophobic methoxyl groups. Elution order is: delphinidin (dpd), cyanidin (cyd), petunidin (ptd), pelargonidin (pgd), peonidin (pnd), and malvidin (mvd). Because of their instability and inherent difficulties for purification, the availability of anthocyanin standards has been limited and they have tended to be expensive. This has hampered the technique from progressing beyond the fingerprint or semiquantitative stages. Fortunately this does not seriously limit the utility of HPLC for qualitative analyses since the anthocyanins of most commodities are well characterized and auxiliary techniques are available for making correct peak assignments (Hong and Wrolstad, 1990a,b). Juices and extracts (e.g., prepared from cranberries, grapes, strawberries) can themselves serve as references for retention indices and spectral characteristics. Commercial sources for purified anthocyanins have recently become more available, which facilitates identification and quantitation (e.g., Extrasynthese and Polyphenols AS).

Critical Parameters and Troubleshooting

The sample preparation procedures and HPLC separations described in this unit are designed for aqueous samples. When investigating the anthocyanin composition of plant materials and solid foods, aqueous extracts can be prepared through acetone extraction as described in *UNIT F2.1*. When methanol extraction is used for anthocyanin isolation (*UNIT F1.1*), the methanol should be nearly completely removed on a rotary evaporator and replaced with water. For isolation of anthocyanins by solid-phase extraction (*UNIT F1.1*), it is critical that the sample be in aqueous solution. Injection of a dilute alcoholic solution (<15%) of anthocyanins may have relatively little effect on chromatographic behavior, since the small injection volume (typically 50 µl) will have a minor influence on the composition of the mobile phase which starts at about 5% organic solvent. Wines can be injected as is, the amount of ethanol in the injected wine sample being too small to have a significant impact on mobile phase composition.

Isolation of anthocyanins by solid-phase extraction (SPE; see Alternate Protocol) prior to injection is not necessary for most analyses. This is particularly the case for separation on Silica C_{18} columns (see Basic Protocol 1); however, in cases where the sample contains considerable degraded or polymeric pigment because of processing or storage abuse, improved chromatographic resolution will be achieved through sample clean-up. The experience of the authors has been that materials containing acylated anthocyanins (e.g., red grapes, red cab-

bage, radishes) and highly complex samples (e.g., blueberries) may also benefit from anthocyanin purification with C_{18} SPE.

These are relatively simple and robust procedures. Some of the factors to keep in mind when working with anthocyanins are excessive heat, light, oxidation, and sample handling, as these factors can alter or destroy these labile compounds. The preparation and HPLC separation of anthocyanins on silica C_{18} columns (see Basic Protocol 1) is the easiest and most robust of the procedures. Typical care in filtering samples and solvents prior to HPLC analysis is about all that is necessary.

Preparation of acylated anthocyanins and anthocyanidins (see Basic Protocol 2 and Alternate Protocol) requires that the SPE cartridges are not overloaded with sample, or that sample is lost because it is not adsorbed onto the SPE cartridge. Also, the sample should not be rotoevaporated to dryness or left at elevated temperature for excessive time periods.

Preparation of anthocyanidins (see Basic Protocol 3) requires that samples be handled quickly and kept cold and in the dark to prevent degradation.

Saponification of acylated anthocyanins and their HPLC separation (see Basic Protocol 3) requires that the samples be thoroughly saponified (longer time if necessary) to differentiate acylated peaks from those that are not.

Table F1.3.4 presents more information on common problems.

Data analysis

The methods described in this unit are qualitative analysis; therefore, results are typically reported as percent total peak area. Chromatographic peaks that are less than 1% of total peak area are generally ignored during data analysis, which is justified since these small peaks do not materially affect the color or composition of a product. The analyses can be performed in a quantitative fashion if the analyst is careful to adjust and record all volumes and quantities during extraction procedures. The use of an authentic external standard such as cyd-3-glu is necessary to determine proper coefficients to use for calculations. Even though the extinction coefficients are different for each anthocyanin (varies with aglycon and sugar substituent), it would be impractical to try and quantitate each peak with it's specific standard; therefore, it is the accepted practice to use the values from a single standard (i.e., cyd-3-glu) and apply those values to all the peaks in a chromatogram. Other useful numbers that are sometimes reported include peak ratios — i.e., (% cyd-3-gal/% cyd-3-arab) or even to sum up various peaks to get the ratios by aglycon cyd/pnd — e.g., (% cyd-3-gal + %cyd-3-glu + % cyd-3-arab)/(% pnd-3-gal + % pnd-3-glu + % pnd-3-arab).

Anticipated Results

Either Basic Protocol 1 or the Alternate Protocol should first be conducted in analyzing an unknown sample. Because of its ease and simplicity, sample preparation of anthocyanins and their HPLC separation on silica C18 columns (see Basic Protocol 1) is usually the preferred choice, unless the presence of acylated anthocyanins is anticipated, in which case the protocol described for acylated anthocyanins is used (see Alternate Protocol). If the anthocyanin profile is inconsistent with previously published chromatograms, or if there are extraneous unidentified peaks, then simplification is recommended (see Basic Protocol 3). Acid hydrolysis will simplify the chroma-

Table F1.3.4 Troubleshooting Guide for HPLC Analysis of Anthocyanins and Anthocyanidins

Problem	Possible cause	Solution
All peaks come out early and/or bunched together	Differences between column elution profiles	Adjust gradient to decrease percentage acetonitrile at early run times.
Not all peaks are eluted by the end of the gradient or they are eluted during the wash out section of the gradient	Differences between column elution profiles	Adjust gradient to increase percentage acetonitrile at later run times, earlier run times, or both.
Two very early peaks, resolved, but shortly after the void volume of the column	Betalains (beet colorant) in the sample	Check the peak spectrum against the literature or standards to see that they are actually anthocyanins.

togram since there are only six anthocyanidins, and few commodities contain more than 2 or 3 of them. This protocol is very complementary to Basic Protocol 1 for investigations on fruit juice authenticity. For example, red raspberry contains cyanidin and pelargonidin but never contains delphinidin, while strawberry should have ~90% pelargonidin, 10% cyanidin, and no other anthocyanidins; cranberry contains about equal amounts of cyanidin and peonidin with traces of delphinidin but never pelargonidin nor malvidin, while grape samples always contain malvidin.

Data interpretation and peak identification are aided when one uses a diode array detector. Acquiring spectral information about the peaks as they elute from the chromatogram allows one to deduce some information about the structure of the peaks, and to determine peak purity and accurate wavelength of maximum absorption. The latter helps to determine the amount of hydroxyl and methoxyl substitution on the B ring (Fig. F1.3.1; Hong and Wrolstad, 1990a,b). The amount and type of phenolic or organic acid acylation can be inferred from the UV spectrum (Hong and Wrolstad, 1990a,b). Figure F1.3.5 shows that acylation can be determined from the $A_{acyl\text{-}max}/A_{vis\text{-}max}$ (Harborne, 1958). The $A_{acyl\text{-}max}$ is the peak in the 310 to 320 nm region. If the ratio is very low, there is no acylation. If it is 0.5 to 0.7 then there is a single acylation, while ratios of 0.8 to 1.1 indicate two acylating groups. Figure F1.3.5 also shows that -3- versus -3,5-glycosylation can be determined from the $A_{440}/A_{vis\text{-}max}$ (Hong and Wrolstad, 1990a,b). If the ratio is greater than ~0.3 the peak is -3-glycosylated. If it is less than 0.2 it is -3,5-diglycosylated.

Spectral information can be used to determine if peaks are acylated. This is determined by a peak in the 310 nm region (Hong and Wrolstad, 1990a,b). If the sample contains acylated peaks, then the longer Alternate Protocol is recommended. Simplification (see Basic Protocol 3) is advised for further characterization of the pigments. One should see elimination (reduction) of those peaks which are acylated anthocyanins, and either an increase in the glycoside peak(s), or the appearance of a new peak if the parent glycosylated pigment is not present in the original sample.

Typical HPLC chromatograms with peak assignments for several common fruits are illustrated in a book chapter by Wrolstad et al. (1994). While most of the chromatograms shown were generated using the Alternate Protocol, the pattern (elution order and relative peak areas) is essentially the same as for Basic

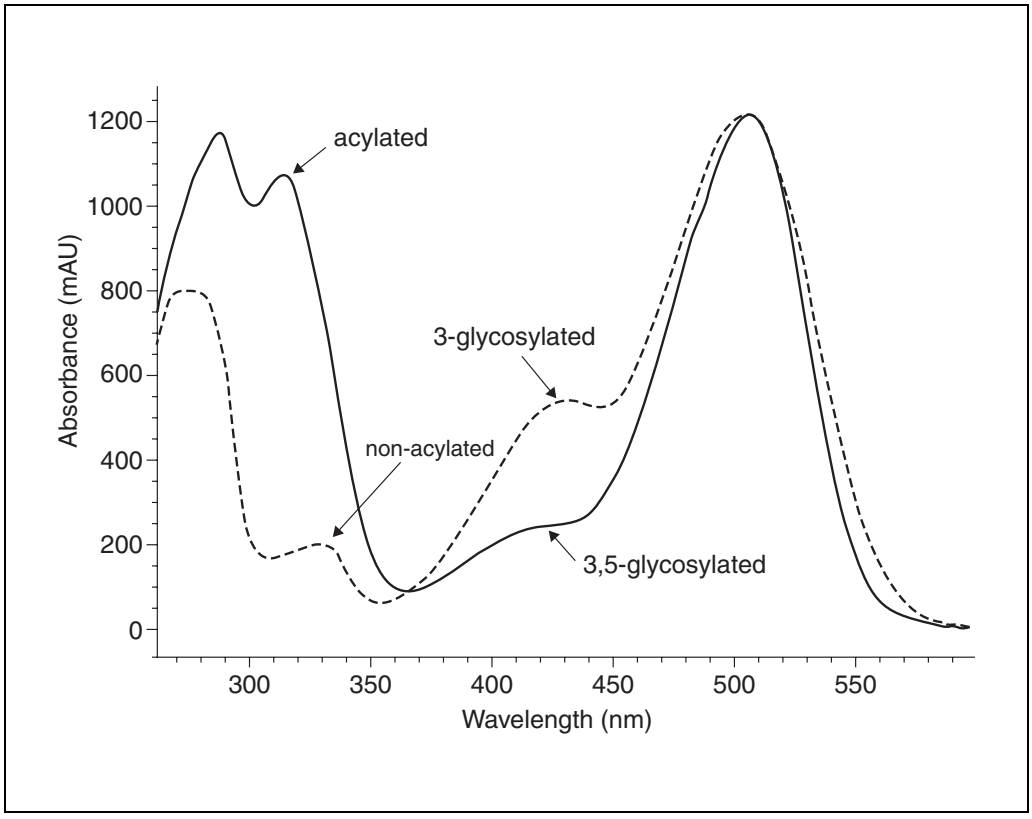


Figure F1.3.5 Spectra of -3-glycosylated (nonacylated; dashed line) and -3,5-glycosylated (acylated; solid line) pigments.

Protocol 1, except that retention times are longer. One should routinely run a sample of known composition to confirm retention times and ensure that the system is running appropriately. This is important as the analysis is quite sensitive to minor changes in solvent composition. Cranberry juice cocktail makes a good reference material that is readily available, inexpensive, and includes peaks over a broad range of retention times. Authentic standards can also be purchased. Cyanidin-3-glucoside is an appropriate choice since it is the most common occurring anthocyanin pigment in nature. Availability of authentic standards makes quantitative analysis of the anthocyanins possible using the external standard method.

Figure F1.3.4 shows HPLC chromatograms for anthocyanidins generated from acid hydrolysis of concord grape and strawberry juices. Extraneous peaks may be present because of incomplete hydrolysis, and degradation and polymerization of the labile aglycons even more of a problem. For acylated anthocyanins, higher yields of anthocyanidins will be achieved if the sample is first saponified (see Basic Protocol 3) and then subjected to acid hydrolysis (see Basic Protocol 2).

Figure F1.3.6 compares the HPLC chromatograms for radish anthocyanin extract before and after saponification. Prior to saponification there are four unique peaks present in the sample. Upon removal of the acylating group(s) from each of the four peaks by Basic Protocol 3, the same parent is formed (pelargonidin-3-sophoroside-5-glucoside), hence only a single peak in the chromatogram after saponification (Giusti and Wrolstad, 1996).

Time Considerations

In Basic Protocol 1 both the sample preparation and analysis are quick and easy. Sample preparation is a matter of a few minutes, while analysis takes 30 to 35 min between injections.

The Alternate Protocol has a longer sample preparation time (15 to 20 min) and a considerably longer analysis time of 75 min between injections.

Basic Protocol 2 takes about an hour to prepare a sample, but by staggering the start of preparation, 2 to 3 samples/hour can be prepared. The limiting factor is getting them analyzed before they degrade, since the analysis time is about 30 min between injections. Remember that it will likely be necessary to prepare a concord grape standard and/or a strawberry standard periodically to confirm retention times.

Basic Protocol 3 sample preparation takes 15 to 25 min with analysis taking either 30 to 35 min if Basic Protocol 1 is used or 75 min if Basic Protocol 2 is used for analysis.

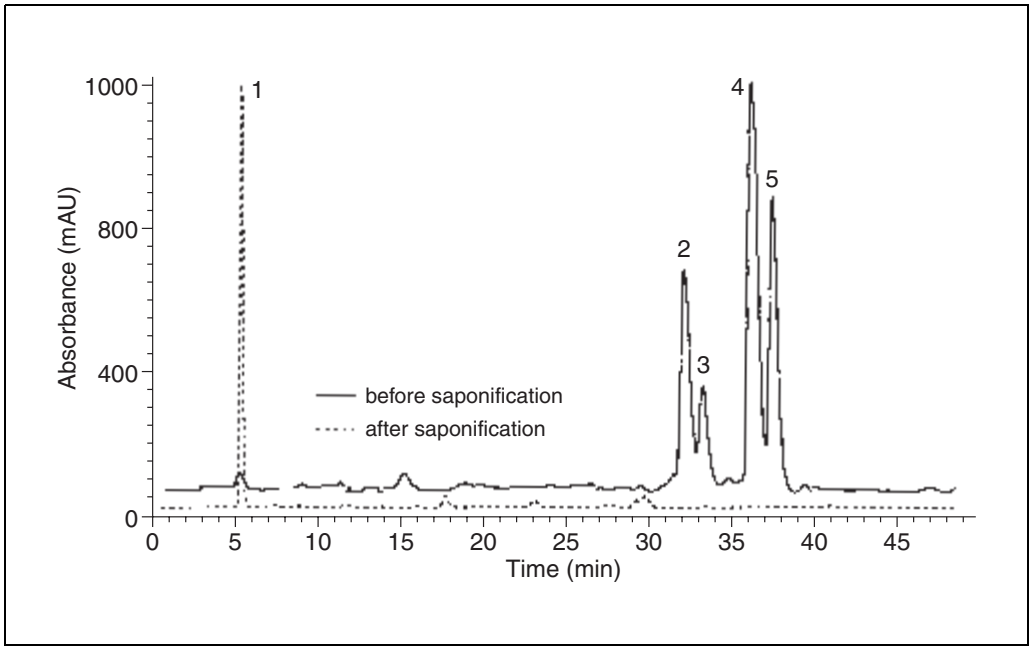


Figure F1.3.6 HPLC chromatograms of radish anthocyanin extract before (solid line) and after (dashed line) saponification. Peak 1: pelargonidin-3-sophoroside-5-glucoside; Peaks 2, 3, 4, 5: pelargonidin-3-sophoroside-5-glucoside acylated with *p*-coumaric (2), ferrulic (3), *p*-coumaric and malonic (4), or ferrulic and malonic acid (5), respectively. Note that the saponified sample has only pelargonidin-3-sophoroside-5-glucoside.

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Key References

Hong and Wrolstad, 1990a. See above.

Methods for systematic identification of anthocyanins are described.

Hong and Wrolstad, 1990b. See above.

The HPLC and spectral characteristics for 10 different anthocyanin extracts and colorants are presented.

Wrolstad et al., 1994. See above.

HPLC chromatograms with peak assignments are given for several commodities along with applications of anthocyanin analyses for determining authenticity of fruit juices.

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Characterization of Anthocyanins by NMR

According to the authors' unpublished files, the structures of more than 620 different anthocyanins have hitherto been elucidated. Individual anthocyanins have diverse impact on color and color stability of many fresh and processed fruits and vegetables, as well as, most probably, potential with respect to health benefits. Thus, the demand for exact structure elucidations has swelled with increased interest for these water-soluble plant pigments, which have colors ranging from salmon-pink through red, violet, and nearly black. In this context, the development of reliable superconducting magnets coupled to application of pulse technique and its associated Fourier transform have made nuclear magnetic resonance spectroscopy (NMR) the most important tool for complete structure elucidation of anthocyanins. The NMR method is especially valuable for determination of the nature of the sugar moieties and the sites of acyl and sugar substitutions. Other methods and instrumentation used for separation and characterization of anthocyanins have been described in *UNITS F1.1 & F.1.2*, as well as *UNIT F.1.3*.

This unit consists of a description of a sequence of NMR experiments (^{1}H, CAPT, DQF-COSY, TOCSY, HSQC, HMBC; see Background Information), which normally will be sufficient to achieve complete assignments of the proton and carbon resonances of anthocyanins (see Basic Protocol), and assumes that sufficient pure anthocyanin material (at least 1 mg) has been obtained. This sequence will also supply enough information to determine the linkage positions between the anthocyanin building blocks. Values for additional parameters typical for the HMBC, HSQC, DQF-COSY, TOCSY, NOESY, and ROESY experiments applied on anthocyanins are included in Table F1.4.1. This unit also describes preparation of anthocyanin NMR samples (see Support Protocol 1), and a method for revealing important steps during recording of a 1-D ^{1}H NMR spectrum (see Support Protocol 2). The latter may be used as a basis for recording ^{13}C NMR spectra, and for initial preparation of two-dimensional homo- and heteronuclear experiments. A discussion concerning 2-D NMR experiments (DQF-COSY, TOCSY, HSQC, HMBC, NOESY, and ROESY), chemical shifts (^{1}H and ^{13}C), and coupling constants (^{1}H-^{1}H) on selected anthocyanins (Table F1.4.3 to Table F1.4.7), as well as other information, is included in the later part of the unit (see Commentary). As an example for analysis, a detailed route for structure elucidation of the major anthocyanin cyanidin 3-*O*-(2″-*O*-β-glucopyranosyl-6′-*O*-α-rhamnopyranosyl-β-glucopyranoside) isolated from tart cherries, *Prunus cerasus*, is described (see Support Protocol 1). An NMR glossary including some common NMR terms is also included in the appendix at the end of this unit.

RECORDING NMR SPECTRA FOR ASSIGNMENTS OF PROTON AND CARBON SIGNALS OF ANTHOCYANINS

BASIC PROTOCOL

Structure elucidation, including NMR, depends on assignments of the proton and carbon resonances. This protocol describes a sequence of NMR experiments which, in most cases, will assign all the proton and carbon resonances of the individual building blocks of an anthocyanin, as well as giving the linkage points between these units.

Various parameters must be taken into account when an NMR experiment is performed. Different manufacturers (e.g., Bruker, Varian, Jeol) label some of these parameters differently; however, the user manuals usually contain enough details about basic acquisition and processing. For the NMR experiments included in this protocol, the authors recommend referring to Braun et al. (1998) for descriptions of the pulse programs and important acquisition and processing parameters. The nomenclature used in this protocol

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follows the Bruker AMX instrument using the UXNMR system; however, a glossary of abbreviations and symbols of other manufacturer dialects (Varian, Jeol) is included as an appendix in this key reference. Table F1.4.1 contains important acquisition parameters. Background information about the same NMR experiments applied on anthocyanins is provided later in the unit (see Commentary).

The proton resonance frequency, not the field strength, is usually applied to classify the NMR machines. Thus, the NMR instrument should be at least 300 MHz or stronger (e.g. the field strength must be 70 kG or stronger) to achieve constructive anthocyanin spectra. Optimal spectral resolution depends on several factors, including the quality of the NMR tubes (Table F1.4.2).

Table F1.4.1 Parameters for 2-D NMR Experiments Performed on a Typical Anthocyanin[a,b]

Experiment	Example	SW (^{13}C) ppm	SW (^{1}H)	Number of scans	Number of FIDs (t_1)	Data points (t_2)	Optimized coupling constants
HMBC	Fig. F1.4.7	160	8.5	2	512	2K	145 Hz ($^{1}J_{CH}$) 8Hz ($^{n}J_{CH}$)
HSQC	Fig. F1.4.6	160	8.5	2	512	2K	145 Hz ($^{1}J_{CH}$)
COSY	Fig. F1.4.3		8.5	2	512	2K	7.5 Hz ($^{3}J_{HH}$ and $^{2}J_{HH}$)
TOCSY	Fig. F1.4.5		9.2	16	512	2K	
NOESY			8.5			2K	
ROESY			8.5			2K	

[a]Abbreviations: SW, sweep width.

[b]Cyanin 3-(2″-glucosyl-6″-rhamnosylglucoside) (**15**; see Fig. F1.4.1), recorded at 600 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DMX 600 instrument.

Table F1.4.2 NMR Sample Tube Requirements for NMR Instruments with Different Field Strengths[a]

NMR instrument	Norell sample tube	Wilmad sample tube
Up to 900 MHz	No. 5020-USP	Wilmad 542
Up to 800 MHz	No. 5010-USP	Wilmad 541
Up to 600 MHz	No. 509-UP	–
Up to 500 MHz	No. 508-UP	Wilmad 535
Up to 400 MHz	No. 507-HP	Wilmad 528
Up to 300 MHz	No. 506-P	Wilmad 597, 526, 527

[a]Data provided by the NMR sample tube producers Wilmad and Norell.

Table F1.4.3 Guide to molecules used in Tables F1.4.4 and F1.4.5[a]

Identifier	Name	Reference
1	Cyanidin 3-arabinoside	Ramstad et al., 1995
2	Delphinidin 3-rhamnoside	Cabrita et al., 2000
3	Delphinidin 3-galactoside	Fossen et al., 1998
4	Pelargonidin 3-glucoside	Pedersen et al., 1993
5	Cyanidin 3-glucoside	Andersen et al., 1991a
6	Peonidin 3-glucoside	Van Calsteren et al., 1991
7	Apigeninidin 5-glucoside	Swinny et al., 2000
8	Petunidin 3-rhamnoside-5-glucoside	Catalano et al., 1998
9	Malvidin 3,5-diglucoside	Andersen et al., 1995
10	Cyanidin 3-(2″-xylosylgalactoside)	Cabrita, 1999
11	Cyanidin 3-(2″-xylosylglucoside)	Andersen et al., 1991a
12	Cyanidin 3-(2″-glucosylgalactoside)	Slimestad and Andersen, 1998
13	Cyanidin 3-(2″-glucosylglucoside)	Slimestad and Andersen, 1998
14	6-Hydroxycyanidin 3-(6″-rhamnosylglucoside)	Nygård et al., 1997
15[b]	Cyanidin 3-(2″-glucosyl-6″-rhamnosylglucoside)	Unpub. observ.
16	Delphinidin 3-(6″-acetylgalactoside)	Fossen et al., 1998
17	Cyanidin 3-(6″-malonylglucoside)	Fossen et al., 1996
18	Delphinidin 3-(2″-galloyl-6″-acetylgalactoside)	Fossen et al., 1998
19[c]	Delphinidin 3-(6″-malonylglucoside)-5-glucoside	Unpub. observ.
20	Petunidin 3-(6″-(4‴″-*E*-*p*-coumaroylrhamnosyl)-glucoside)-5-glucoside	Andersen et al., 1991b
21	Malvidin 3-(6″-(4‴″-*E*-caffeoylrhamnosyl)-glucoside)-5-glucoside.	Slimestad et al., 1999

[a]All samples dissolved in CD_3OD acidified with CF_3COOD or DCl at 25°C.

[b]See Figure F1.4.1.

[c]See Figure F1.4.4.

Table F1.4.4 ^{1}H NMR Spectral Data for Selected Anthocyanins[a]

Molecule[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Aglycone	cy	dp	dp	pg	cy	pn	ap	pt	mv	cy	cy	cy	cy	OHcy	cy	dp	cy	dp	dp	pt	mv
3							8.16														
4	9.02	9.04	9.07	9.10	9.10	9.00	9.24	9.05	9.24	9.00	8.96	9.05	9.06	8.99	8.90	9.00	8.99	8.99	8.90	8.97	9.09
6	6.75	6.74	6.74	6.74	6.76	6.67	7.06	7.07	7.17	6.73	6.73	6.72	6.74		6.72	6.75	6.74	6.73	7.06	7.06	7.12
8	6.98	6.94	6.95	6.96	6.98	6.94	7.14	7.08	7.26	6.95	6.94	6.95	6.98	7.20	6.92	6.96	6.95	6.90	7.05	7.06	7.12
2′	8.16	7.65	7.88	8.61	8.14	8.13	8.38	7.67	8.12	8.11	8.05	8.13	8.11	8.07	8.00	7.87	8.07	7.64	7.77	7.94	8.12
3′				7.10			7.10														
5′	7.10			7.10	7.11	7.07	7.10			7.08	7.05	7.12	7.12	7.09	7.08		7.08				
6′	8.40	7.65	7.88	8.61	8.31	8.23	8.38	7.60	8.12	8.33	8.28	8.27	8.28	8.27	8.21	7.87	8.33	7.64	7.77	7.78	8.12
OMe						3.99		4.02	4.09											4.02	4.10
3gly/5glc	ara	rha	gal	glc	glc	glc	5glc	rha	glc	gal	glc	gal	glc	glc	glc	gal	glc	gal	glc	glc	glc
1″	5.37	5.82	5.36	5.37	5.38	5.33	5.21	5.98	5.45	5.48	5.53	5.49	5.54	5.42	5.45	5.34	5.36	5.63	5.58	5.69	5.59
2″	4.13	4.35	4.11	3.76	3.78	3.64	m	4.32	3.75	4.32	4.07	4.36	4.12	3.76	4.11	4.11	3.79	5.78	3.86	3.85	3.81
3″	3.88	4.02	3.78	3.68	3.65	3.58	m	4.02	3.65	4.01	3.90	3.99	3.85	3.66	3.83	3.78	3.58	4.07	3.78	3.75	3.69
4″	4.08	3.66	4.05	3.58	3.56	3.45	3.47	3.59	3.51	4.08	3.62	4.07	3.62	3.50	3.55	4.04	3.43	4.13	3.55	3.61	3.57
5″/5A″	4.10	3.73	3.85	3.70	3.67	3.60	m	3.60	3.61	3.95	3.71	3.90	3.65	3.83	3.77	4.15	3.88	4.27	4.01	3.97	3.92
6A″/5B″	3.87	1.37	3.86	4.05	4.02	3.91	3.97	1.37	4.03	3.92	4.03	3.90	4.00	4.14	4.11	4.43	4.65	4.52	4.56	4.16	4.12
6B″			3.86	3.86	3.82	3.71	3.76		3.79	3.92	3.85	3.90	3.81	3.68	3.67	4.37	4.37	4.44	4.45	3.85	3.82
2″xyl/6″rha										xyl	xyl			rha	rha					rha	rha
1‴										4.81	4.87			4.77	4.72					4.83	4.82
2‴										3.28	3.29			3.92	3.85					3.93	3.91
3‴										3.43	3.43			3.78	3.68					3.94	3.92
4‴										3.52	3.53			3.41	3.39					4.99	5.02
5‴/5A‴										3.76	3.80			3.65	3.63					3.86	3.82
6‴/5B‴										3.13	3.16			1.28	1.20					1.09	1.12
5glc/2″glc								5glc	5glc			2″**glc**	2″**glc**		2″**glc**				5glc	5glc	5glc
1‴								5.28	5.26			4.81	4.85		4.85				5.29	5.31	5.29

continued

Table F1.4.4 ^{1}H NMR Spectral Data for Selected Anthocyanins[a], *continued*

Molecule[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
2‴								3.69	3.73			3.30	3.29		3.28				3.79	3.82	3.80
3‴								3.59	3.59			3.40	3.36		3.38				3.69	3.71	3.77
4‴								3.49	3.44			3.32	3.32		3.32				3.55	3.65	3.64
5‴								3.61	3.69			3.02	3.03		3.01				3.87	3.72	3.71
6A‴								3.99	4.01			3.50	3.54		3.54				4.06	4.06	4.02
6B‴								3.79	3.82			3.50	3.54		3.54				3.85	3.92	3.89
2″gall/4‴″cou/4‴″caf																		gall		cou	caf
α																				6.27	5.69
β																				7.61	6.88
2																		7.06		7.46	7.22
3																				6.88	
5																				6.88	6.86
6																		7.06		7.46	7.00
6″ace/6″mal																ace	mal	ace	mal		
2‴″/2‴																2.13	3.44	2.18	3.46		

[a]Abbreviations: ap, apigeninidin; ara, arabinose; ace, acetyl; caf, caffeoyl; cou, coumaroyl; cy, cyanidin; dp, delphinidin; gal, galactose; gall, galloyl; glc, glucose; gly, glycoside; mal, malonyl; mv, malvidin; pg, pelargonidin; pn, peonidin; pt, petunidin; OHcy, 6-hydroxycyanidin; rha, rhamnose; xyl, xylose.

[b]Refer to Table F1.4.3 for molecule identities and references.

Table F1.4.5 ^{13}C NMR Spectral Data for Selected Anthocyanins[a,b]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21
Aglycone	cy	dp	dp	pg	cy	pn	ap	pt	mv	cy	cy	cy	cy	OHcy	cy	dp	cy	dp	pt	mv
2	164.59	164.49	164.49	163.85	164.36	164.19	173.8	164.14	164.5	164.15	164.29	164.20	164.34	162.28	163.03	164.38	164.30	164.51	163.79	164.56
3	145.56	144.51	145.96	145.31	145.64	145.49	112.0	145.49	146.8	145.32	145.40	145.35	145.33	145.85	144.28	145.98	145.55	145.62	146.09	146.25
4	136.46	135.51	136.61	137.08	137.03	137.35	149.9	133.90	136.2	136.23	136.34	136.58	136.80	134.39	135.01	135.68	136.69	135.34	134.08	134.99
5	159.10	159.11	159.03	157.48	159.55	159.29	158.6	156.97	157.4	159.63	159.56	159.16	159.16	141.95	158.04	159.38	159.70	159.12	156.89	156.75
6	103.42	103.38	103.29	103.49	103.50	103.42	105.5	105.43	105.9	103.46	103.63	103.34	103.34	135.62	102.67	103.30	103.43	103.30	105.54	105.61
7	170.44	170.34	170.38	170.59	170.56	170.70	172.1	169.48	169.8	170.22	170.71	nd	170.23	159.37	169.50	170.24	170.49	170.33	169.73	169.90
8	95.24	95.08	95.03	95.28	95.19	95.24	98.5	97.33	97.6	95.14	95.23	95.10	95.15	95.24	94.43	95.13	95.26	95.18	97.55	97.64
9	157.64	157.71	157.72	157.44	157.75	157.86	160.2	156.69	156.9	157.45	157.75	157.58	157.60	151.56	156.51	157.64	157.71	157.66	156.61	153.33
10	113.29	113.36	113.29	113.39	113.45	113.63	114.5	113.31	113.6	113.19	113.42	113.28	113.30	114.04	112.21	113.87	113.23	112.97	113.03	113.31
1′	121.26	120.00	120.07	120.61	121.31	121.14	121.6	119.68	119.7	121.27	121.36	121.28	121.27	121.38	120.25	120.01	121.18	119.53	119.58	119.50
2′	118.48	112.11	112.62	135.67	118.56	115.19	134.1	108.56	111.1	118.66	118.70	118.69	118.58	118.11	117.62	112.71	118.37	112.68	109.52	110.97
3′	147.51	147.73	147.56	117.91	147.41	149.51	118.9	149.84	149.9	147.37	147.79	147.36	147.40	147.19	146.43	147.57	147.44	147.38	149.76	149.89
4′	155.94	144.89	144.71	166.57	155.78	156.37	168.3	147.61	147.1	155.75	156.16	155.64	155.67	154.97	154.80	144.91	155.86	144.79	147.75	146.75
5′	117.46	147.73	147.56	117.91	117.48	117.55	118.9	145.76	149.9	117.44	117.55	117.53	117.45	117.51	116.69	147.57	117.39	147.38	146.16	149.89
6′	128.58	112.11	112.62	135.67	128.22	128.84	134.1	113.90	111.1	128.57	128.80	128.09	128.22	127.68	127.50	112.71	128.47	112.68	114.03	110.97
OCH_3						56.91		57.16	57.4										57.22	57.26
3gly/5glc	ara	rha	gal	glc	glc	glc	glc	rha	glc	gal	glc	gal	glc	glc	glc	gal	glc	gal	glc	glc
1″	104.13	102.64	104.63	103.70	103.79	103.77	103.1	102.14	102.7	102.16	101.69	102.40	101.98	103.39	100.90	104.06	103.58	102.41	102.54	102.79
2″	72.10	71.55	72.16	74.77	74.80	74.88	75.0	71.50	74.5	80.12	81.91	80.67	82.33	74.68	80.89	71.89	74.64	73.20	74.73	74.78
3″	73.71	72.31	74.87	78.13	78.13	78.11	78.2	72.36	78.7	75.08	78.47	74.75	77.84	77.98	76.79	74.60	77.90	72.74	78.23	78.25
4″	68.68	73.31	70.14	71.06	71.11	71.12	71.5	73.29	71.4	70.01	71.06	69.85	71.22	71.27	69.98	70.31	71.31	70.48	71.31	71.32
5″	66.75	72.16	77.80	78.73	78.79	78.87	79.9	72.25	77.7	77.66	78.90	77.53	77.69	77.42	76.22	75.15	75.93	75.46	77.69	77.66
6″		17.95	62.35	62.33	62.39	62.33	62.8	18.05	62.5	62.29	62.55	62.28	62.27	67.87	66.75	65.20	65.47	65.17	67.33	67.08
6″rha/2″xyl										xyl	xyl			rha	rha				rha	rha
1‴/1⁗										106.04	105.89			102.10	101.18				102.15	102.13
2‴/2⁗										75.78	75.98			71.85	70.95				72.09	72.09
3‴/3⁗										77.85	78.17			72.32	71.50				70.39	70.34
4‴/4⁗										70.94	71.25			73.84	72.90				75.40	75.28
5‴/5⁗										67.13	67.37			69.80	68.95				67.88	67.85
6‴/6⁗														17.90	17.09				17.85	17.84
5glc/2″glc								5glc	5glc			2″glc	2″glc		2″glc				5glc	5glc
1‴								102.50	104.4			105.35	104.98		103.82				102.69	103.07
2‴								74.59	74.9			75.97	75.87		75.00				74.84	74.78
3‴								77.82	78.5			77.68	77.98		76.75				77.88	77.90

continued

Table F1.4.5 ^{13}C NMR Spectral Data for Selected Anthocyanins[a,b], *continued*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21
4‴								71.04	71.1			71.14	70.82		70.20				70 95	70 97
5‴								78.63	79.0			77.89	78.58		77.10				78.64	78.73
6‴								62.32	62.4			62.27	62.27		61.32				62.13	62.16
2″gall/4‴″cour																				
4‴″caf																		gall	cou	caf
α																			114.98	114.99
β																			147.09	147.36
1																		120.82	127.16	127.73
2																		110.50	131.31	115.38
3																		146.30	116.92	146.75
4																		140.15	161.26	149.59
5																		146.30	116.92	116.50
6																		110.50	131.31	122.99
C=O																		168.14	169.09	168.98
6″acyl																ace	mal	ace		
1‴″/1‴																172.76	166.65	172.83		
2‴″/2‴																20.69		20.74		
3‴																	170.19			

[a]See Table F1.4.3 for definitions of anthocyanin identifiers and references, and Table F1.4.4 for abbreviations (nd, not detected).

[b]Data for **19** not included.

Table F1.4.6 Typical ^{1}H-^{1}H Coupling Constants of the Most Common Anthocyanidins[a]

	Pelargonidin	Cyanidin	Peonidin	Delphinidin	Petunidin	Malvidin
H-4	*s* (*b*)	*d*, 0.9 Hz	*d*, 0.9 Hz	*d*, 0.9 Hz	*s* (*b*)	*s* (*b*)
H-6	*d*, 1.9 Hz	*d*, 1.9 Hz	*d*, 1.9 Hz	*d*, 1.9 Hz	*d*, 1.9 Hz	*d,* 1.9 Hz
H-8	*dd*, 0.9 Hz, 1.9 Hz	*dd*, 0.9 Hz, 1.9 Hz	*dd*, 0.9 Hz, 1.9 Hz	*dd*, 0.9 Hz, 1.9 Hz	*d* (*b*), 1.9 Hz	*d* (*b*), 1.9 Hz
H-2′	*'d'*, 8.7Hz	*d*, 2.3 Hz	*d*, 2.3 Hz	*s*	*d*, 2.1 Hz	*s*
H-3′	*'d'*, 8.7 Hz					
H-5′	*'d'*, 8.7 Hz	*d*, 8.7 Hz	*d*, 8.7 Hz			
H-6′	*'d'*, 8.7 Hz	*dd*, 2.3 Hz, 8.7 Hz	*dd*, 2.3 Hz, 8.7 Hz	*s*	*d*, 2.1 Hz	*s*
OCH_3			*s*		*s*	*s*

[a]Abbreviations: *b,* broad; *d*, doublet; *'d'*, semidoublet; *s*, singlet.

Table F1.4.7 Typical ^{1}H-^{1}H Coupling Constants for the Monosaccharides Commonly Identified in Anthocyanins[a]

	Glucopyranose	Galactopyranose	Rhamnopyranose	Xylopyranose	Arabinopyranose
1″	*d*, 7.7 Hz	*d*, 7.7 Hz	*d*, 1.5 Hz	*d*, 7.7 Hz	*d*, 6.2 Hz
2″	*dd*, 7.7 Hz, 9.4 Hz	*dd*, 7.7 Hz, 9.6 Hz	*dd*, 1.5 Hz, 3.3 Hz	*dd*, 7.7 Hz, 9.2 Hz	*dd*, 6.2 Hz, 8.1 Hz
3″	*t*, 9.4 Hz	*dd*, 9.6 Hz, 3.4Hz	*dd*, 3.3 Hz, 9.3 Hz	*t*, 9.2 Hz	*d* (*b*) 8.1 Hz
4″	*t*, 9.4 Hz	*dd*, 3.4 Hz, 1.0 Hz	*t*, 9.4 Hz	*ddd*, 10.3 Hz, 9.2 Hz, 5.5 Hz	*m*
5A″	*ddd*, 2.0 Hz, 6.7 Hz, 9.4 Hz	*ddd*, 1.0 Hz, 3.7 Hz, 8.3 Hz	*dd*, 9.5 Hz, 6.2 Hz	*dd*, 11.4 Hz, 5.5 Hz	*dd*, 15.0 Hz, 4.1 Hz
5B″				*dd*, 11.4 Hz, 10.3 Hz	*d* (*b*), 15.0 Hz
6A″	*dd*, 12.0 Hz, 2.0 Hz	*dd*, 11.8 Hz, 8.3 Hz	*d*, 6.2 Hz		
6B″	*dd*, 12.0 Hz, 6.7 Hz	*dd*, 11.8 Hz, 3.7 Hz			

[a]Abbreviations: *b*, broad; *d*, doublet; *s*, singlet; *t*, triplet.

Materials

NMR instrument of 300 MHz or stronger and appropriate sample tube (Table F1.4.2)

Additional reagents and equipment for preparation of anthocyanin NMR samples (see Support Protocol 1), recording the 1-D ^{1}H NMR spectrum (see Support Protocol 2), and recording the 2-D ^{1}H-^{13}C HMBC, 2-D ^{1}H-^{13}C HSQC, 2-D ^{1}H-^{1}H DQF-COSY, 2-D ^{1}H-^{1}H TOCSY, 2-D ^{1}H-^{1}H NOESY, and 1-D ^{13}C CAPT spectrums (Braun et al., 1998)

1. Prepare the NMR sample (see Support Protocol 1).

   In this protocol, 20 mg cyanidin 3-(2″-glucosyl-6″-rhamnosylglucoside) dissolved in 0.5 ml CD_3OD/CF_3COOD (95:5 v/v) represents a typical sample. Using a relatively concentrated sample (~50 mM) facilitates shorter experiment time and better quality of the 1-D ^{13}C and 2-D NMR spectra.

2. Set the temperature (usually 298K) on the NMR instrument, and allow time for the instrument to achieve the chosen temperature.

3. Insert the NMR sample into the sample tube.

4. Read a shim file suitable for the NMR solvent and solvent volume (~40 mm) being used.

   The shimming of the NMR sample must be optimized to improve the magnetic field homogeneity (for procedure, see Braun et al., 1998). A shim file stored on the NMR computer is normally used as a starting point for the shimming procedure. This shim file should be regularly updated from the optimized shim settings of the sample. On high-field superconducting NMR spectrometers, it is essential that the probe-head be correctly tuned to the observed frequency to obtain a favorable signal-to-noise ratio. Furthermore, impedance matching of the network must also be performed. In the more recent NMR spectrometers, wobbling functions are programmed in the software, making tuning and matching a very easy process.

5. Record the 1-D ^{1}H NMR spectrum (see Support Protocol 2).

6. Record the 2-D ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) spectrum (Braun et al., 1998, pp. 489-492).

7. Record the 2-D ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectrum (Braun et al., 1998, pp. 497-500).

8. Record the 2-D ^{1}H-^{1}H double quantum filtered correlation spectroscopy (DQF-COSY) spectrum (Braun et al., 1998, pp. 481-484).

9. Record the 2-D ^{1}H-^{1}H total correlation spectroscopy (TOCSY) spectrum (Braun et al., 1998, pp. 501-504).

10. Record the 2-D ^{1}H-^{1}H nuclear Overhaüser enhancement spectroscopy (NOESY) spectrum (Braun et al., 1998, pp. 405-408).

11. Record the 1-D ^{13}C compensated attached proton test (CAPT) spectrum (Braun et al., 1998, pp. 165-167).

    ^{1}H and ^{13}C NMR spectral data for various anthocyanins are given in Tables F1.4.4 and F1.4.5. Typical ^{1}H-^{1}H coupling constants for common anthocyanidins and their monosaccharides are given in Tables F1.4.6 and F1.4.7.

SUPPORT PROTOCOL 1

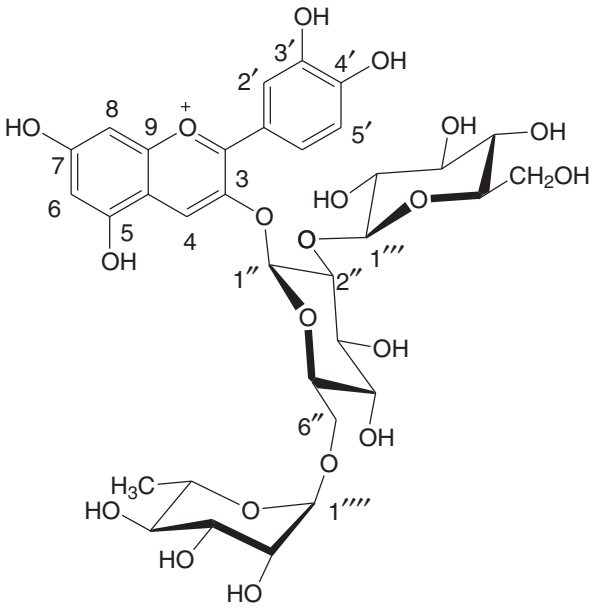
PREPARATION OF AN ANTHOCYANIN NMR SAMPLE

This protocol describes isolation of anthocyanins, using cherries as an example, as well as how a pure anthocyanin, cyanidin 3-(2″-glucosyl-6″-rhamnosylglucoside) (**S.15**; Fig. F1.4.1) is treated before NMR experiments are performed. In this protocol, 20 mg **S.15** is dissolved in 0.5 ml of 95:5 (v/v) CD_3OD/CF_3COOD. Refer to *UNIT F1.1* for further details on extraction, purification, and isolation of anthocyanins. Common NMR solvents for anthocyanins are given in Table F1.4.8.

Materials

Cherries (*Prunus cerasus*)
99.9:0.1 (v/v) methanol/HCl
Ethyl acetate
Amberlite XAD-7 resin (Sigma)
1% (v/v) trifluoroacetic acid (TFA) in methanol
20:79.5:0.5 and 40:59.5:0.5 (v/v) methanol/H_2O/TFA
Nitrogen gas
95:5 (v/v) CD_3OD/CF_3COOD

80×5–cm chromatography column
90×5–cm Sephadex LH-20 column
NMR sample tube (Table F1.4.2) with plug
Glass pipets
~100°C oven



15

Figure F1.4.1 The structure of the major anthocyanin, cyanidin 3-*O*-(2″-*O*-β-glucopyranosyl-6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), isolated from tart cherries, *Prunus cerasus*. Structure number **15** corresponds to ^1H and ^{13}C NMR data in Tables F1.4.4 and F.1.4.5.

Table F1.4.8 Common NMR Solvents for Anthocyanins

Solvent	Secondary reference for ^{1}H NMR and ^{13}C NMR
95:5 (v/v) CD_3OD/CF_3COOD	Residual solvent peak (CHD_2OD) at δ 3.40 and the solvent peak at δ 49.0 from TMS for ^{1}H and ^{13}C, respectively
80:20 (v/v) DMSO-d_6/CF_3COOD	Residual solvent peak (DMSO-Hd_5) at δ 2.49 and the solvent peak at d 39.6 from TMS for ^{1}H and ^{13}C, respectively

1. Extract anthocyanins from 700 g cherries using 900 ml of 99.9:0.1 (v/v) methanol/HCl three times.
2. Purify the concentrated extract by partitioning against an equal volume of ethyl acetate three times, followed by adsorption chromatography using an 80 × 5–cm column packed with Amberlite XAD-7 resin, which has been washed in advance with 2 liters water. Elute using 1% trifluoroacetic acid methanol.
3. Isolate pure anthocyanins on a 90 × 5–cm Sephadex LH-20 column using a solvent gradient of 20:79.5:0.5 (v/v) to 40:59.5:0.5 (v/v) methanol/water/TFA.
4. Evaporate the acidified methanol from the sample under nitrogen (2 hr).
5. Dry the glass equipment (i.e., NMR sample tube without plug and glass pipettes) for 2 hr in an ~100°C oven.
6. Dry the sample, pipet tips, and the NMR sample tube plug 2 hr in a desiccator under vacuum at room temperature.
7. Dissolve the sample in 0.5 ml of 95:5 (v/v) CD_3OD/CF_3COOD.
8. Transfer the sample to an NMR sample tube using a dry pipet.
9. Plug the NMR sample tube and seal with Parafilm.

RECORDING OF THE 1-D ^{1}H NMR SPECTRUM AND INITIAL PREPARATIONS FOR 2-D EXPERIMENTS

SUPPORT PROTOCOL 2

This protocol describes how a 1-D ^{1}H NMR spectrum is recorded. This type of spectrum may provide useful information about the identity of the aglycone, number of attached sugar units, and presence or absence of acyl moieties. This procedure may also be used as the basis for recording ^{13}C NMR spectra, and for initial preparations of 2-D homo- and heteronuclear experiments. See Basic Protocol for materials.

1. If it hasn't been done already, prepare the NMR sample, set the temperature, and read a shim file as described (see Basic Protocol, steps 1 to 4).
2. Adjust the lock-phase and check for ^{2}H lock signal saturation.
3. Tune and match the probe head's ^{1}H and ^{13}C channels, respectively.

   Tuning and matching of the ^{13}C channel is only necessary for experiments involving ^{13}C (e.g., HSQC, HMBC).

4. Define a new data set and read a suitable parameter file for the 1-D ^{1}H experiment. Record a preliminary 1-D ^{1}H spectrum with one scan and a relatively large sweep width (at least 10 ppm).

   From this preliminary spectrum, the sweep width of the 1-D ^{1}H spectrum must be defined so that all anthocyanin signals are included. The anthocyanin ^{1}H and ^{13}C signals are normally found in the spectral regions 0.5 to 9.5 ppm and 10 to 180 ppm, respectively.

5. Establish the 90° pulses for ^{1}H and ^{13}C, respectively, by the following procedure.
   a. Perform on-resonance 90° pulse calibration by defining the middle of the spectrum (O1) at the strongest spectrum signal—e.g., the water peak or the solvent peak in the preliminary ^{1}H spectrum (or the solvent peak in the ^{13}C NMR spectrum).
   b. Record a new spectrum at the ~90° pulse. Fourier-transform and correct the phase.
   c. Determine the ~360° pulse by multiplying the ~90° pulse by four ($4 \times 90° = 360°$).

      Thereafter, record the spectrum with the ~360° pulse.
   d. Record a spectrum with the slightly changed ~360° pulse.
   e. Repeat substep b using the ~360° pulse. Continue repeating until the pulse is exactly 360°.

      At this point, the intensity of the selected on-resonance signal is ~0 (i.e., the positive and negative amplitudes of the signal are equal).
   f. Divide the exact 360° pulse by 4 to find the exact 90° pulse.
6. Adjust the receiver gain by using the RGA command (for a Bruker instrument).
7. Select number of scans (usually 8 to 256) for the experiment.

   The optimum number of scans for a given sample depends on several factors (e.g., sample concentration, field strength of the instrument) and must be determined individually.
8. Record the final 1-D ^{1}H NMR spectrum.

COMMENTARY

Background Information

The name anthocyanin (anthos meaning flower and kyanos meaning blue) was originally used to describe the pigment in blue cornflower (*Centaurea cyanus*). In a checklist from 1988 (Harborne and Grayer, 1988), 256 different anthocyanidin and anthocyanin structures were reported from a variety of plant sources with colors ranging from salmon-pink through red and violet, to nearly black. In 1999, the number of anthocyanins was increased to 453 (Harborne and Baxter, 1999), and the number today is >620 (unpub. observ.). This dramatic increase may in general reflect the fact that recent improvements in methods and instrumentation have made it easier to use smaller quantities of material to achieve results at increasing levels of precision. The progress with respect to complete structure elucidations is mainly caused by application of soft mass spectrometry techniques such as fast atomic bombardment (FAB; Self, 1987), tandem mass spectrometry (Glaessgen et al., 1992; Giusti et al., 1999), electrospray ionization (ESI; Kondo et al., 1994; Piovan et al., 1998), and matrix-assisted laser desorption/ionization (MALDI; Sporns and Wang, 1998; Sugui et al., 1999), and above all, various NMR techniques.

Today, it is possible to make complete assignments of all proton and carbon atoms in the NMR spectra of most isolated anthocyanins. These assignments are normally based on chemical shifts (δ) and coupling constants (J) observed in 1-D ^{1}H and ^{13}C NMR spectra (Fig. F1.4.2), combined with correlations observed as cross-peaks in various homo- and heteronuclear 2-D NMR experiments (see below for details on COSY, TOCSY, HSQC, HMBC, NOESY, and ROESY).

The advance in computing power has been an important factor for the success of the more advanced NMR techniques. Running many scans and accumulating the data may enhance weak signals, because baseline noise, which is random, tends to cancel out. One of the main advantages to be gained from signal averaging combined with the use of Fourier transform methods and high field magnets is the ability to obtain ^{13}C NMR spectra. This isotope of carbon exists in low abundance (1.108%) compared to the essentially 100% abundance of ^{1}H. The NMR sensitivity also depends on the magnetogyric constants, which for ^{13}C is only a quarter of the value of ^{1}H. Thus, the sample amount required for ^{13}C NMR spectra is about ten times that for ^{1}H NMR spectra, and the number of scans are normally 100 times higher.

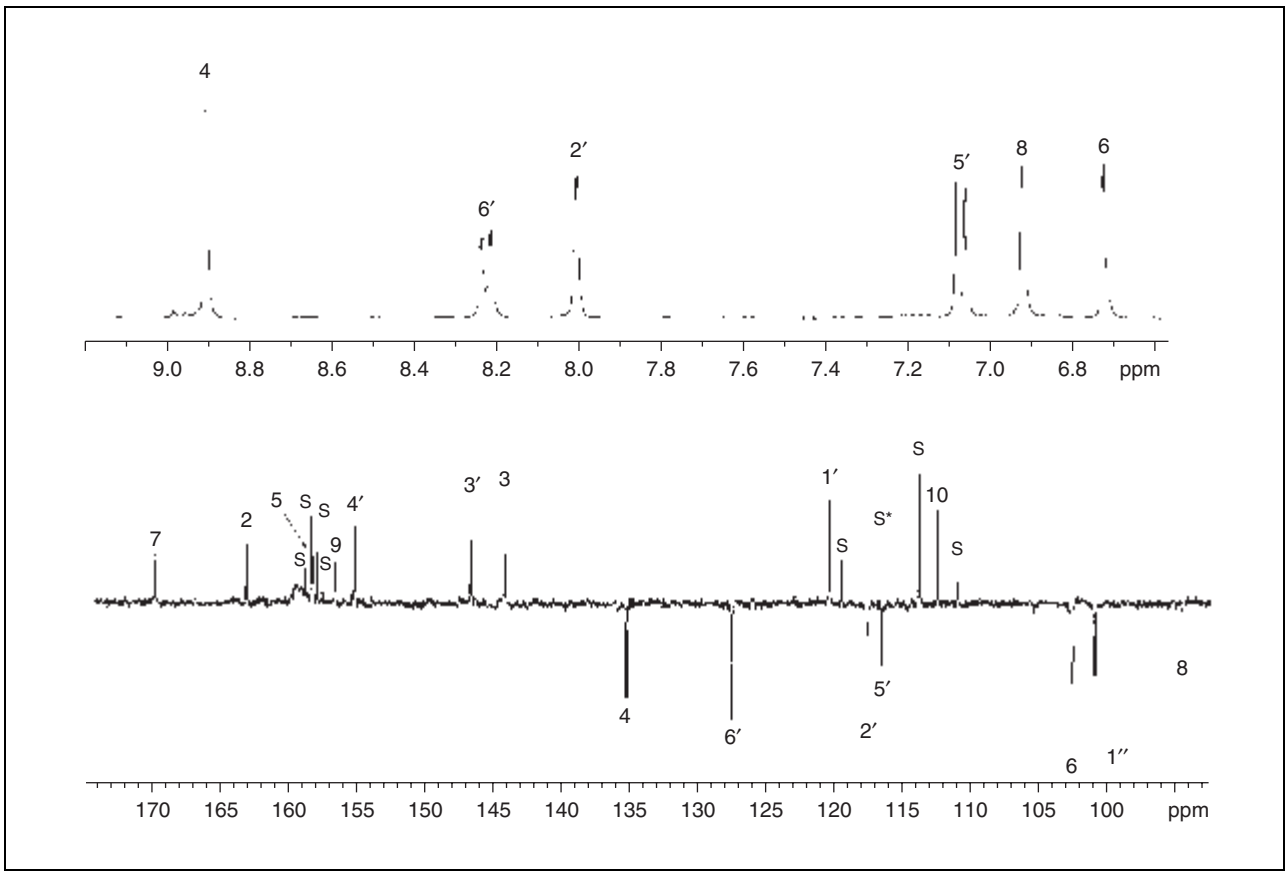


Figure F1.4.2 The aromatic region of the 1-D ^{1}H NMR of cyanidin gives rise to a characteristic splitting pattern (upper spectrum). The lower spectrum shows the aromatic region of the 1-D ^{13}C compensated attached proton test (CAPT) NMR spectrum of cyanidin. This spectrum contains all the fifteen ^{13}C resonances of the aglycone in addition to solvent signals (labeled S) and the anomeric sugar proton labeled 1″. In this spectrum, the ^{13}C nuclei which have a proton attached are represented with resonances pointing downwards, while the quaternary ^{13}C nuclei are pointing upwards. The ^{1}H NMR experiment was obtained within 25 sec, while the CAPT experiment was obtained within 1 hr 46 min.

Several experiments are suitable for recording anthocyanin 1-D ^{13}C NMR spectra. For example, in Fig. F1.4.2 the authors have used a compensated attached proton test (CAPT; Torres et al., 1993; Braun et al., 1998).

Introduction to 2-D NMR experiments

The purpose of the standard 1-D ^{1}H NMR experiment is to achieve structure-related information about sample protons (i.e., chemical shifts, spin-spin couplings, and integration data) describing the relative number of protons. Applied to anthocyanins, this information may help to identify the aglycone (anthocyanidin), number of monosaccharides present, and anomeric configuration of the monosaccharides. However, for most anthocyanins, the information gained by a standard 1-D ^{1}H NMR experiment is insufficient for complete structure elucidation. In recent years, various 2-D NMR experiments have evolved as the most powerful tools for complete structure elucidation of anthocyanins.

Two-dimensional NMR spectra are mainly produced as contour maps. These maps may be best imagined as looking down on a forest where all the trees (representing peaks in the spectrum) have been chopped off at the same fixed height. 2-D NMR spectra are produced by homonuclear (^{1}H-^{1}H) and heteronuclear (^{1}H-^{13}C) experiments.

Homonuclear ^{1}H-^{1}H-correlated NMR experiments, like the ^{1}H-^{1}H DQF-COSY and ^{1}H-^{1}H TOCSY experiments shown in Figs. F1.4.3 and F1.4.5, generate NMR spectra in which ^{1}H chemical shifts along both axes are correlated with each other. Values on the diagonal of these spectra correspond to chemical shifts, (e.g., trees; see above paragraph) that would have been shown in a 1-D ^{1}H NMR experiment. It is the off-diagonal spots called cross-peaks that present information which is new. These cross-peaks arise from coupling interactions between different ^{1}H nuclei. A cross-peak observed above the diagonal will normally also be found below the diagonal, thus producing a nearly

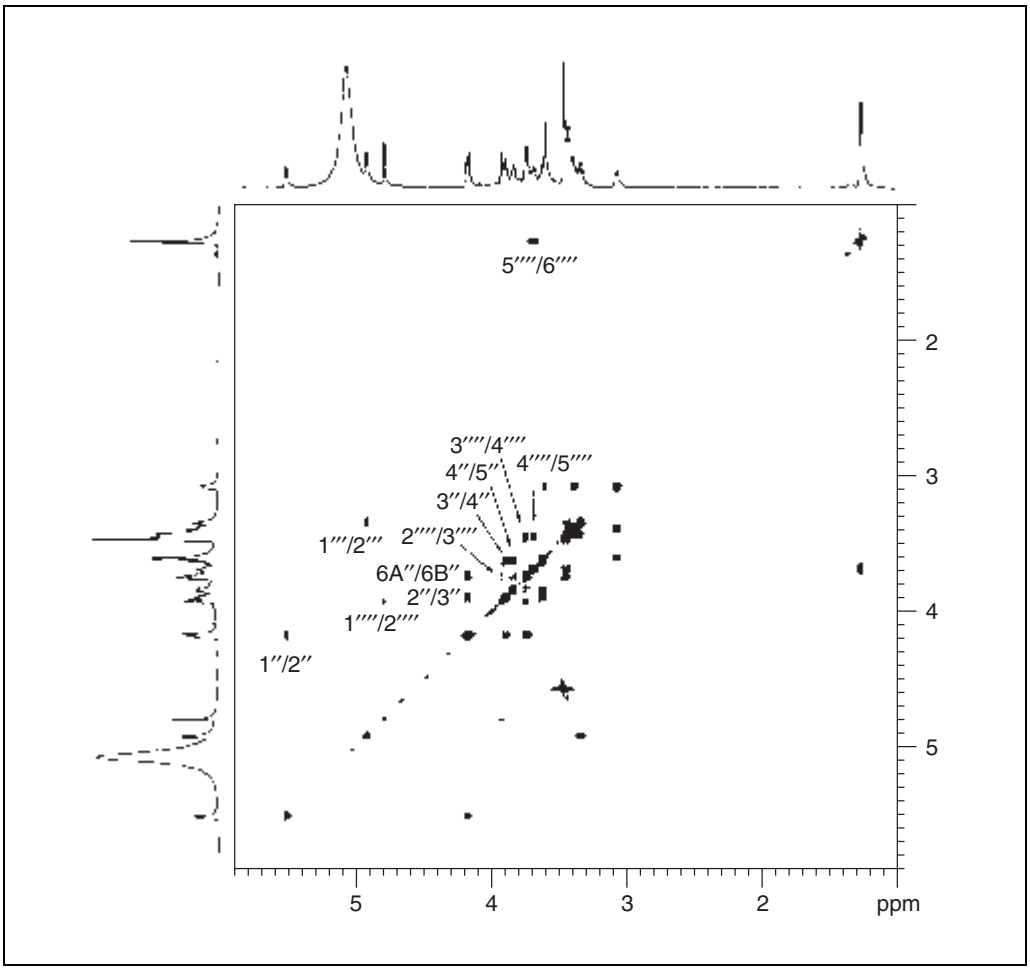
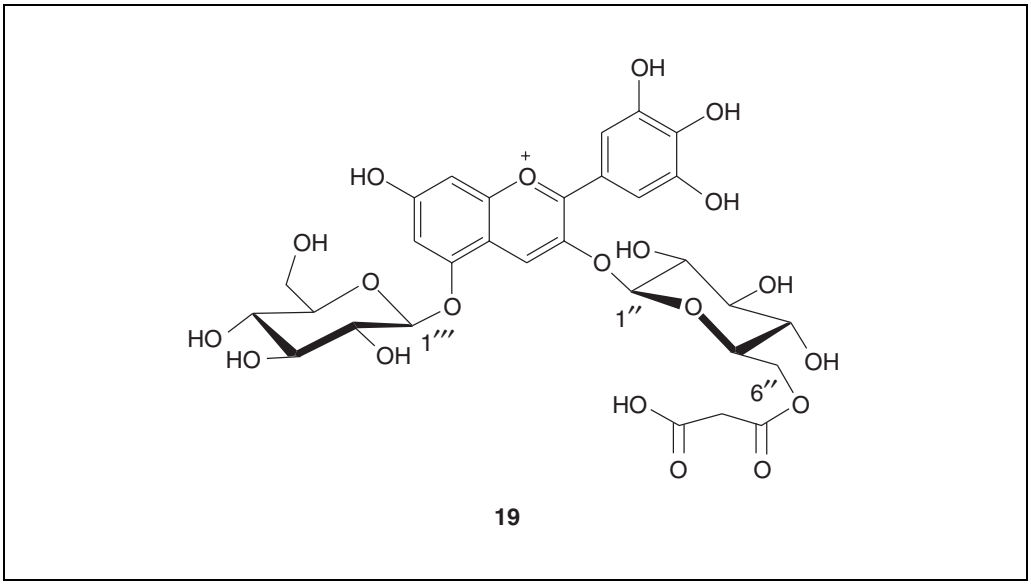


Figure F1.4.3 ^{1}H-^{1}H DQF-COSY spectrum of the sugar region of **15** (Fig. F1.4.1) showing assignments of the individual ^{1}H resonances of the anthocyanin sugar units by $^{3}J_{HH}$ and $^{2}J_{HH}$ interactions. The cross-peaks involving the rhamnose and one of the glucose units are labeled. The COSY spectrum was obtained within 39 min.



19

Figure F1.4.4 The structure of the acylated anthocyanin delphinidin 3-*O*-(6′′-malonyl-β-glucopyranoside)-5-β-glucopyranoside, isolated from *Aster novi belgii.* Structure number **19** corresponds to ^{1}H NMR data in Table F1.4.4.

symmetrical spectrum. The 1-D ^{1}H NMR spectra may be placed as projections along the top and left part of the 2-D NMR spectra, as shown in Fig. F1.4.3 and Fig. F1.4.5. The 1-D spectra are positioned along the axes to help assignments of the cross-peaks. The ^{1}H-^{1}H NOESY and ^{1}H-^{1}H ROESY experiments described below are other examples of homonuclear NMR experiments.

Heteronuclear NMR experiments are represented by the HSQC and HMBC spectra in Fig. F1.4.6 and Fig. F1.4.7. The ^{13}C NMR spectrum (or ^{13}C-projection) is displayed along one axis, and the ^{1}H NMR spectrum (or ^{1}H-projection) along the other. The ^{1}H-^{13}C correlations are shown as cross-peaks in the spectrum. Contrary to homonuclear NMR experiments, there exist no diagonal and only one cross-peak for each correlation.

COSY

The part of the 1-D ^{1}H NMR spectrum of cyanidin 3-*O*-(2″-glucopyranosyl-6′-*O*-α-rhamnopyranosyl-β-glucopyranoside), **15**, between 3.0 and 4.5 ppm, which represents the majority of sugar protons, is too crowded to present interpretable information. However, the double-quantum filtered correlation spectroscopy (^{1}H-^{1}H DQF-COSY) spectrum of this region (Fig. F1.4.3) makes it possible to assign all the protons of the rhamnose and the two glucose units of this compound. The DQF-COSY spectrum shows couplings between neighboring protons ($^2J_{HH}$, $^3J_{HH}$ and $^4J_{HH}$) revealed as cross-peaks in the spectrum. Interpretation may start from the anomeric H-1″ signal on the diagonal at 5.45 ppm, which is detected 1 to 2 ppm downfield to the rest of the sugar signals. A ruler can then be placed horizontally through the cross-peak labeled 1″/2″.

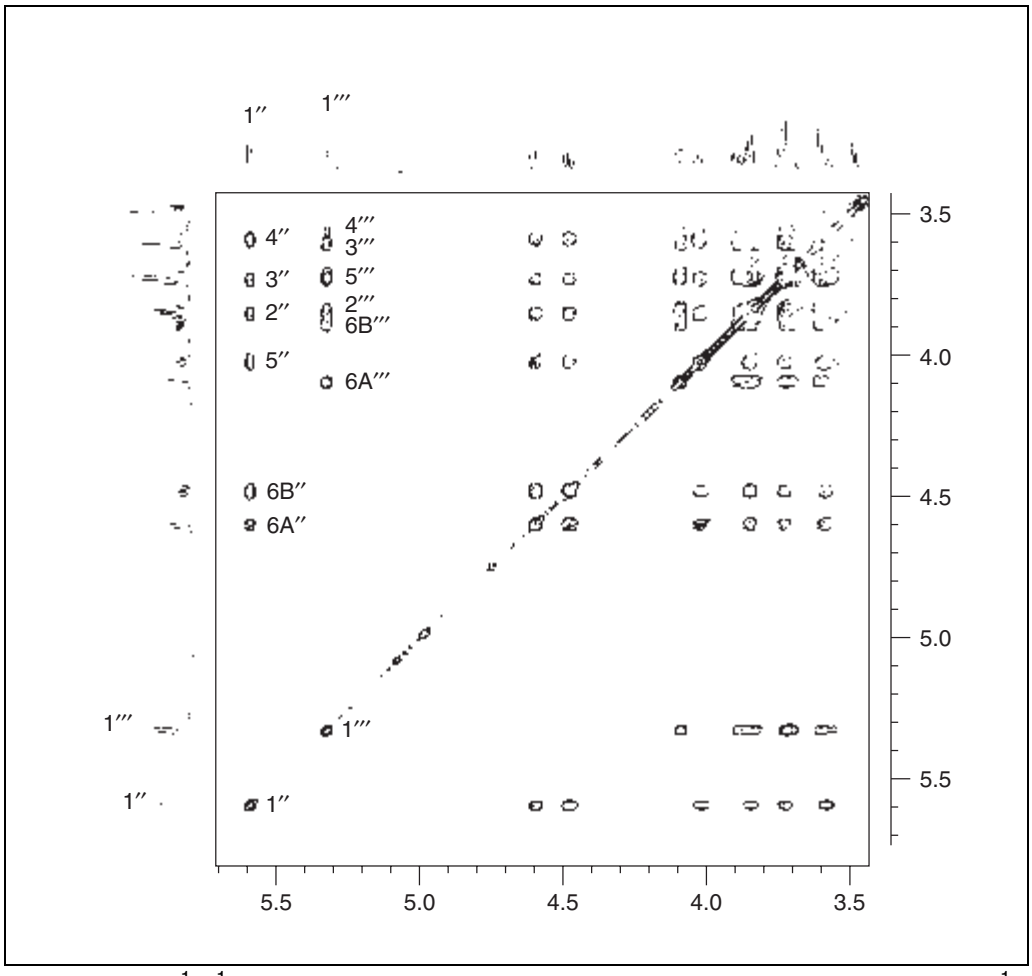


Figure F1.4.5 ^{1}H-^{1}H TOCSY spectrum of the sugar region of **19** (Fig. F1.4.4) showing all the ^{1}H resonances belonging to each spin system (e.g., to each sugar unit). In this spectrum it is possible to determine the chemical shift of all the protons belonging to each of the sugar units of **19**. The cross-peaks of each sugar unit are assigned in the spectrum. The TOCSY experiment was obtained within 5 hr 18 min.

This cross-peak represents the coupling between H-1″ and the proton located on the neighbor carbon (H-2″). The chemical shifts of H-2″ is located where the ruler intersects the diagonal (4.11 ppm). When the ruler is moved vertical through the H-2″ position on the diagonal, it intersects the cross-peak labeled 2″/3″. Thereafter the ruler is located horizontally through this cross-peak, and the intersection with the diagonal gives the chemical shift of H-3″ (3.83 ppm). In a similar manner, it is possible to assign all the protons of the monosaccharides of **15** (Table F1.4.4).

The ^{1}H-^{1}H DQF-COSY experiment is a modification of the standard ^{1}H-^{1}H COSY experiment. The chief advantage of the double-quantum filtered technique is that noncoupled proton signals are eliminated. This technique is especially useful for eliminating the water signal, which may overlap with anthocyanin sugar signals.

TOCSY

The 2-D homonuclear ^{1}H-^{1}H total correlation spectroscopy (TOCSY) experiment (Fig. F1.4.5) identifies protons belonging to the same spin system. As long as successive protons are coupled with coupling constants >5 Hz, magnetization is transferred successively over up to five or six bonds. The presence of heteroatoms, such as oxygen, usually disrupts TOCSY transfer. Since each sugar ring contains a discrete spin system separated by oxygen, this experiment is especially useful for assignments of overlapped anthocyanin sugar protons in the 1-D ^{1}H NMR spectrum. As seen in Fig F1.4.5, the labeled cross-peaks reveal how all the protons within each of the two sugar rings of **19** are correlated. Be aware that the cross-peak intensity is not an indicator of the distance between the protons involved, and that all expected correlations may not appear in a TOCSY spectrum due to the selected mixing time. To

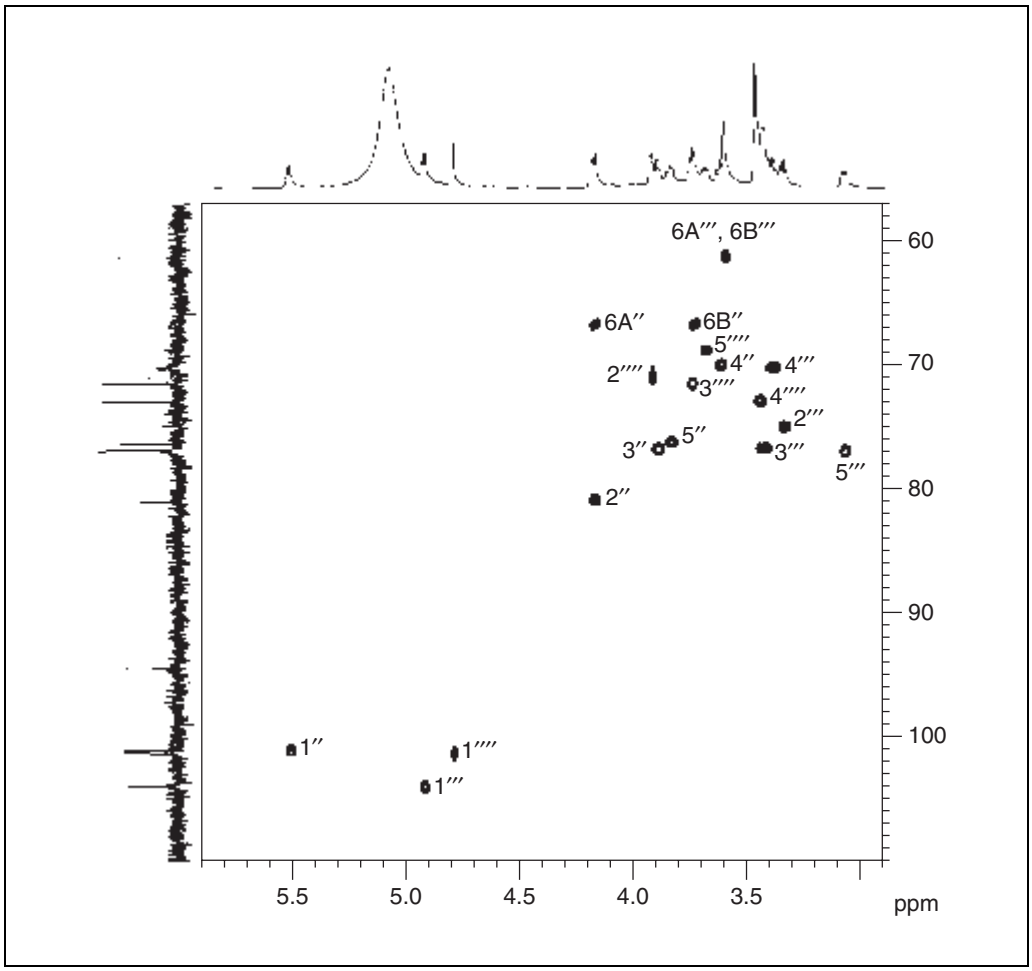


Figure F1.4.6 ^{1}H-^{13}C HSQC spectrum of the sugar region of **15** showing all the $^{1}J_{CH}$ correlations, and thus all the ^{1}H and ^{13}C chemical shifts of the three sugar units, but the cross-peak of the methyl group of rhamnose (H6‴″/C6‴″) at 1.2/17.1 ppm, which occurs beyond the presented region. The HSQC was obtained within 38 min.

avoid this latter problem, it may help to record a second spectrum with another mixing time.

In the 1-D TOCSY experiment, the resonances of one proton are selected and this signal is transferred in a stepwise process to all protons that are J-coupled to this proton. Instead of cross-peaks, magnetization transfer is seen as increased multiplet intensity. Thus, this 1-D TOCSY spectrum looks like a normal ^{1}H NMR spectrum including only the protons that belong to the same spin system as the chosen proton. The 1-D TOCSY experiment version is especially useful to determine coupling constants. For anthocyanins having several overlapping spin systems (e.g., several similar sugar units), additional experiments such as 2-D HSQC-TOCSY may be necessary for complete assignments.

HSQC and HMBC

The proton signals of the three monosaccharides of **15** have previously been assigned by a combination of the ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY experiments. The one-bond ^{1}H-^{13}C correlations observed in the heteronuclear single quantum coherence (HSQC) spectrum of the sugar region of **15** (Fig. F1.4.6), allow the assignment of the corresponding sugar ^{13}C signals. The one-bond ^{1}H-^{13}C coupling constants of anthocyanins, $^{1}J_{CH}$, are observed between 125 and 175 Hz (Pedersen et al., 1995).

The heteronuclear multiple bond correlation (HMBC) experiment correlates proton nuclei with carbon nuclei that are separated by more than one bond. In Fig. F1.4.7 the $^{3}J_{CH}$ and $^{2}J_{CH}$ couplings dominate. Major applications related to anthocyanins include the assignment of resonances of nonprotonated carbon nuclei of the

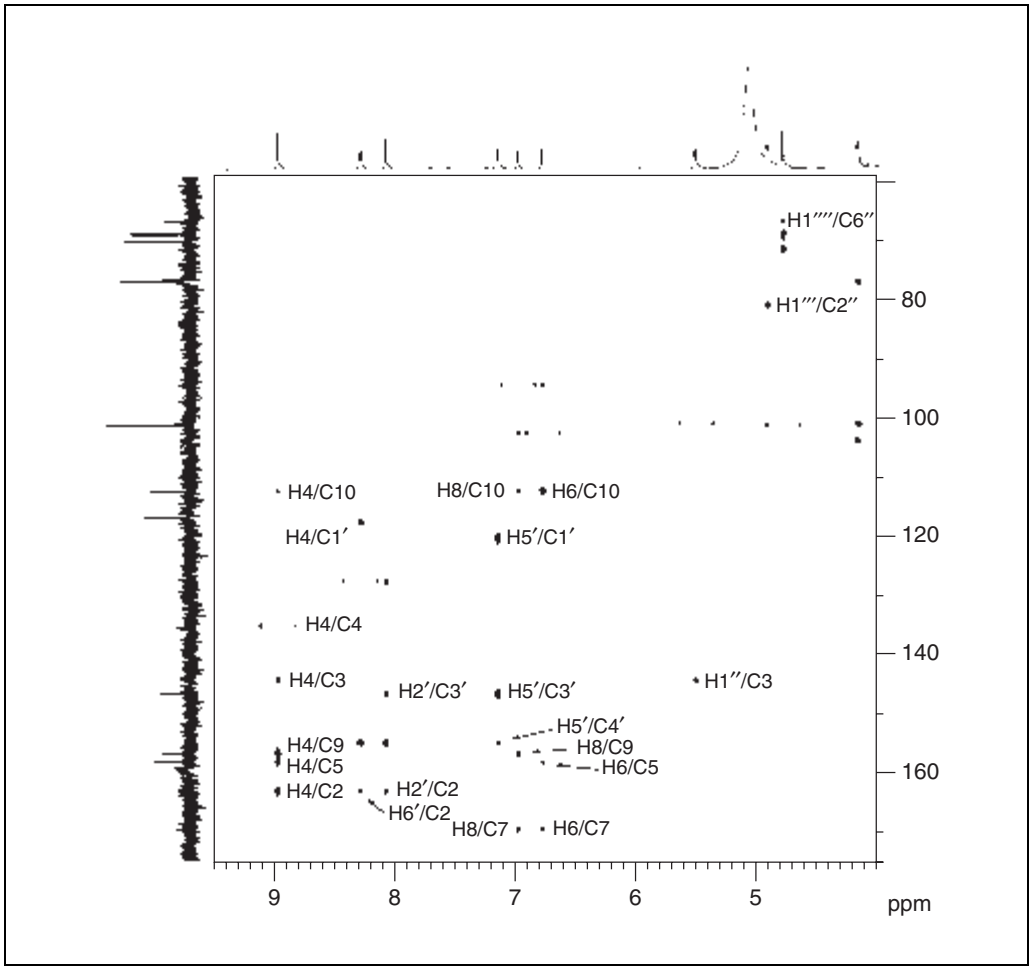


Figure F1.4.7 ^{1}H-^{13}C HMBC spectrum of **15** revealing all ^{1}H-^{13}C long-range couplings of the aglycone. Some important couplings of the sugar units are also labeled: The cross-peak H1‴/C3 (5.45/144.28 ppm), which shows the linkage between the aglycone and one of the glucose units, and the cross-peaks H1‴/C-2″ (4.85/80.89) and H1⁗/C-6″ (4.72/66.75) showing the linkages between the inner glucose unit and the 2″-xylosyl and 6″-rhamnosyl units, respectively. The HMBC experiment was obtained within 40 min.

aglycone (anthocyanidin) and potential acyl group(s). Since long-range correlation of protonated carbon resonances also occur to carbon nuclei that are separated by nonprotonated carbons or other heteronuclei like oxygen, the experiment provides valuable information about the linkage points between the anthocyanin building blocks—aglycone, sugar unit(s), and acyl moieties (Fig. F1.4.7). The intensity of the cross-peaks generated by this experiment is related to the size of the multiple-bond coupling constant, $^{n}J_{CH}$, and the choice of the delay. However, there is no simple relationship between the size of $^{n}J_{CH}$ and the number of intervening bonds, which means that careful analysis of the spectrum in combination with other data is required. One common example of this problem is observed in the aromatic anthocyanin region, where some $^{2}J_{CH}$ are too small to be detected as cross-peaks, while the $^{3}J_{CH}$ are large, resulting in intense cross-peaks.

In the HSQC and HMBC experiments the ^{1}H chemical shifts constitute the natural frequency dimension (called F2), while the ^{13}C chemical shifts constitute the artificial frequency dimension (called F1). The ^{13}C dimension is made by the delays, the ^{13}C pulses and the $^{1}J_{CH}$ couplings. During ^{1}H acquisition in the HSQC experiment, ^{13}C decoupling is applied (garp decoupling). Contrary to the HSQC experiment, decoupling is not applied in the gs-HMBC experiment. In the latter experiment, $^{2}J_{CH}$ and $^{3}J_{CH}$ are favored, and $^{1}J_{CH}$ couplings are suppressed by a low pass filter in the HMBC pulse sequence (Braun et al., 1998). However, the $^{1}J_{CH}$ is not fully eliminated, and some $^{1}J_{CH}$ are observed as symmetrical doublets (Fig. F1.4.7). The one-bond filter has a delay that is matched to the inverse of $^{1}J_{CH}$. The $^{1}J_{CH}$ may also be entered as 145 Hz in this experiment. The HSQC and HMBC experiments are the modern version of the corresponding HETCOR and COLOC experiments (Braun et al., 1998). The two former experiments are more sensitive than the latter since ^{1}H FIDs are acquired, and not ^{13}C FIDs as in the two latter experiments.

NOESY

Couplings don't necessarily have to occur through bonding. Protons that are close to each other in space may be observed as cross-peaks in a nuclear Overhaüser enhancement spectroscopy (NOESY) spectrum. Thus, the more sensitive NOESY experiment proves to be an alternative technique to HMBC for determination of some linkages within an anthocyanin. When a sugar is attached to the aglycone 3-position, a cross-peak between H-4 and the anomeric proton is normally observed. Anomeric protons of monosaccharides attached to the aglycone 5- and 3′-positions show similar cross-peaks to H-6 and H-2′, respectively, while the anomeric proton of a sugar attached to the 7-position will exhibit cross-peaks to both H-6 and H-8.

Cross-peaks observed in a NOESY spectrum may reveal both intra- and intermolecular distances between anthocyanin protons (Nerdal et al., 1992). This type of information has been used for depiction of the association mechanism involving anthocyanins (Nerdal and Andersen, 1992; Houbiers et al., 1997; Gakh et al., 1998; Giusti et al., 1998). Furthermore, based on relative integration of the volume of cross-peaks in the NOESY spectrum, 3-D distance information can be estimated. Cross-peaks corresponding to two protons with a known distance are used as references for the distance calculations. Thus, intermolecular association of two anthocyanin molecules (petanin) have been evidenced by NOESY NMR experiments and distance geometry calculations (Nerdal and Andersen, 1992).

ROESY

Similar to the NOESY experiment the ^{1}H-^{1}H rotational nuclear Overhaüser effect spectroscopy (ROESY) experiment is useful for determination of the signals arising from protons which are close in space, but not necessarily connected by chemical bonds. A ROESY spectrum yields through-space correlations via the rotational nuclear Overhaüser effect (ROE). When one multiplet is irradiated, the intensities of multiplets arising from nearby nuclei are affected. Similar to the NOESY experiment, the ROESY spectrum contains a diagonal and cross-peaks. The diagonal consists of the 1-D spectrum. The cross-peaks indicate an ROE effect between two multiplets. ROESY is especially useful for cases where NOESY signals are weak, because they are near the transition between negative and positive, which often may be the case for medium-sized organic molecules like anthocyanins. ROESY cross-peaks are always negative. The ROESY (and NOESY) experiment also yields cross-peaks arising from chemical exchange. Exchange peaks and TOCSY-type artifacts are always positive so they can be told apart from ROESY correlations.

Assignments of NMR signals

To show how the structure of a relatively complex anthocyanin is completely elucidated by the above-mentioned NMR spectroscopic techniques, a detailed route applied on the major anthocyanin, cyanidin 3-*O*-(2″-*O*-β-glucopyranosyl-6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), **15**, isolated from tart cherries, *Prunus cerasus,* is described below.

Assignments of the protons and carbons of the aglycone

The downfield part of the ^{1}H NMR spectrum of **15** (Fig. F1.4.2) shows six resonances in accordance with the aglycone cyanidin (see Table F1.4.4). The singlet at 8.90 ppm is typical for H-4 of most aglycones (Table F1.4.5). The signals at 8.00, 7.08, and 8.21 ppm are assigned to H-2′, H-5′ and H-6′, respectively, based on their coupling constants (Table F1.4.6). The signals at 8.00 and 7.08 ppm are two doublets with $^4J_{HH}$ and $^3J_{HH}$ of 1.6 and 8.6 Hz, respectively, while the signal at 8.21 ppm is a double doublet with a *meta*-coupling to H-2′ ($^4J_{HH}$ = 1.6 Hz) and an *ortho*-coupling to H-6′ ($^3J_{HH}$ = 8.6 Hz). These couplings are thereafter confirmed by their $^4J_{HH}$ and $^3J_{HH}$ cross-peaks in the DQF-COSY spectrum. The two signals at 6.72 and 6.92 ppm assigned to H-6 and H-8, respectively, have a small *meta*-coupling to each other. In many ^{1}H NMR spectra, this coupling is not obvious; however, it is normally recognized as $^4J_{HH}$ cross-peaks in their COSY and TOCSY spectra when their chemical shift difference is significant. It is nevertheless more common to assign H-6 and H-8 from the $^1J_{CH}$ cross-peaks in the HSQC spectrum, since C-8 normally has its chemical shift around 95 ppm, while C-6 is found around 102 ppm (Table F1.4.5).

After the chemical shifts of the protons of **15** have been assigned, the chemical shifts of the corresponding carbons are assigned from the HSQC experiment. The remaining problem of assigning the quaternary carbon atoms is addressed by the HMBC experiment, which is optimized for $^2J_{CH}$ and $^3J_{CH}$ couplings. A reasonable starting point is at H-4 (8.90 ppm), which couples to seven carbon atoms (Fig. F1.4.7). The strongest cross-peaks are the $^3J_{CH}$ couplings to C-2, C-5, and C-9, respectively. The assignment of C-2 at 163.03 ppm is deduced via its $^3J_{CH}$ couplings to H-4, H-2′ and H-6′, while the remaining signals at 158.04 ppm (C-5) and 156.51 ppm (C-9) are assigned by their additional $^2J_{CH}$ couplings to H-6 and H-8, respectively. The $^2J_{CH}$ coupling to H-4 and the $^3J_{CH}$ coupling to the anomeric proton at 5.45 ppm firmly assign C-3 to the signal at 144.28 ppm. The location of C-10 at 112.21 ppm is deduced from its $^3J_{CH}$ couplings to both H-6 and H-8. The response at 120.25 ppm is assigned to C-1′ by its $^3J_{CH}$ coupling to H-5′ and its $^4J_{CH}$ coupling to H-4. Both C-4′ and C-3′ couples with H-2′ and H-5′, however, only C-4′ (not C-3′) couples with H-6′ (Fig. F1.4.7). The remaining assignment of C-7 to 169.50 ppm is based on its $^2J_{CH}$ to both H-6 and H-8.

Assignments of the protons and carbons of the sugars

The anomeric proton and carbon signals appear considerably downfield of the other sugar resonances, and thus the three cross-peaks at δ5.45/100.90, δ4.85/103.82 and δ4.72/101.18 in the HSQC spectrum of **15** (Fig. F1.4.6) together with integration data, indicate three monosaccharides. The spectral region between δ81 and δ60 in the CAPT spectrum show fourteen resonances which, together with the three anomeric carbon resonances and the methyl signal at δ1.20/17.09, are in agreement with three hexoses. Starting from the doublet at 4.72 ppm, the observed cross-peak with the signal at 3.85 ppm in the DQF-COSY permit assignment of H-2‴′(Fig. F1.4.3). The chain of coupled protons H-2‴′, H-3‴′, H-4‴′, H-5‴′, and H-6‴′ is thereafter assigned using the same spectrum. Subsequently, the chemical shifts of the corresponding carbon atoms (Table F1.4.5) are assigned from the HSQC experiment, which, together with ^{1}H-^{1}H coupling constants, are in agreement with a α-linked rhamnopyranosyl. Similarly, the protons of the two other sugar units are assigned by a "sequential walk" through the cross-peaks in the DQF-COSY spectrum. In cases where several protons show similar chemical shifts, the relationships are supported by the total correlation spectroscopy (TOCSY) experiment, which gives cross-peaks between the anomeric protons and all the protons in the same sugar unit. The chemical shifts and the ^{1}H-^{1}H coupling constants of the sugars of **15** (Table F1.4.4, Table F1.4.5 & Table F1.4.7) agree with one α-rhamnopyranosyl and two β-glucopyranosyl units.

Determination of the linkage points

The HMBC spectrum of **15** (Fig. F1.4.7) reveals the H-1″/C-3 cross-peak at δ5.45/144.28 ppm establishing that the glucosyl is attached to the aglycone 3-position (Fig. F1.4.1). In the same spectrum, the rhamnosyl and the other glucosyl unit are found to be

attached to the 6″- and 2″-positions, respectively, by the cross-peaks at δ4.72/66.75 (H-1‴/C-6″) and δ4.85/80.89 (H-1‴/C-2″). Thus, the identity of **15** is found to be cyanidin 3-*O*-(2″-*O*-β-glucopyranosyl-6″-*O*-α-rhamnopyranosyl-β-glucopyranoside).

The binding sites of the sugars of the anthocyanidins may also be derived from the ROESY or NOESY experiments, which reveal neighborship through space. Strong cross-peaks between an anomeric proton and H-4 will, for instance, indicate that a sugar moiety is connected to the aglycone 3-position. Pronounced downfield shift effects may also confirm the linkage positions of the sugar units.

Critical Parameters and Troubleshooting

A successful structure elucidation of anthocyanins by NMR depends on several factors, including relatively high sample purity and stability. The authors recommend sample purity above 80%, as indicated by an HPLC chromatogram recorded at 280 nm, which will reveal most aromatic compounds in the sample. However, be aware that the molar absorptivities of different aromatic compounds may vary significantly. In addition to aromatic impurities, the sample may contain aliphatic impurities, which may interfere with interpretation of the sugar region of the NMR spectra in particular. The purity of many NMR samples will normally be improved by Sephadex LH-20 column chromatography (e.g., using 39.6:0.4:60 methanol/TFA/H_2O as the eluent).

Each anthocyanin may occur in several equilibrium forms. The use of small amounts of acids is necessary to keep the anthocyanin in the flavyllium cationic form, which is reckoned to be the most stable equilibrium form; however, it is well known that anthocyanin hydrolysis may occur under acidic conditions. The hydrolysis rate will be diminished by low storage temperatures and acid concentrations. Mineral acids (like DCl) should be replaced with organic acids (like CF_3COOD). In recent years it has been recognized that dicarboxylic acid moieties like malonyl may be esterified by an alcoholic solvent (e.g., methanol) under acidic conditions. Thus, the authors have experienced modification of many malonylated anthocyanins both during the isolation procedure and during storage of the NMR samples (Fossen et al., 2001). When recording multidimensional NMR spectra, a 1-D proton NMR spectrum should be recorded prior to and after each experiment to reveal potential changes within the sample. The authors recommend using the same sample for all NMR experiments despite potential modification during storage. Between the experiments, the NMR solution should be kept in a −20°C freezer. For long-term storage (i.e., months), the sample should be dried.

A common problem with respect to NMR spectroscopy on anthocyanins is the relatively slow exchange of the H-6 and H-8 aglycone protons with deuterium in acidified deuterated solvents. Especially when weak anthocyanin samples are involved, this exchange may prevent detection of correlations to C-6 and C-8 in the HSQC experiment and important long-range correlations involving the same protons in the HMBC experiment. As a rough guide, approximately half of the H-6 and H-8 protons are exchanged after 15 hr storage of an anthocyanin dissolved in 19:1 (v/v) CD_3OD/CF_3COOD, room temperature. Therefore, the authors suggest running HMBC and HSQC among the first NMR experiments.

It is very difficult to achieve ^1H NMR spectra of anthocyanins without an intense "water peak" around 5 ppm, which may overlap with signals commonly representing anomeric protons. After some hours of storage in the acidified deuterated solvent, this peak tends to migrate upfield ~0.4 ppm. It is therefore advised to record ^1H NMR spectra immediately after preparation to reveal peaks which may be hidden under the "water peak," and to repeat this procedure after several hours as well.

Anticipated Results

The proton and carbon chemical shifts of twenty-one and twenty different anthocyanins are presented in Table F1.4.4 and Table F1.4.5, respectively. These anthocyanins are chosen to illustrate the chemical shifts of the majority of anthocyanin building blocks reported. The linkage positions of the various anthocyanin building blocks may be conspicuous through shift comparison. However, be aware of shift effects caused by variation in solvent, pigment concentration and temperature. Table F1.4.6 contains typical ^1H-^1H coupling constants of the most common anthocyanidins.

Table F1.4.7 contains typical ^1H-^1H coupling constants for the monosaccharides commonly identified in anthocyanins. When the ring size of the anthocyanin monosaccharides has been reported, all but one have been reported as pyranoses (arabinofuranosyl has been identified in zebrinin; Idaka et al., 1987) . When examined, the anomeric configurations of the glucosyl, galactosyl, xylosyl and glucuronyl

Table F1.4.9 Time Requirement for NMR Experiments on a 50 mM Solution of Cyanidin 3-(2″-Glucosyl-6″-Rhamnosylglucoside)

NMR experiment	Number of scans	Number of experiments	Experiment time
1-D ^{1}H	4	1	25 sec
1-D ^{13}C CAPT	2000	1	1hr 46 min
2-D ^{1}H-^{1}H DQF-COSY	2	512	39 min
2-D ^{1}H-^{1}H TOCSY	8	256	1hr 56 min
2-D ^{1}H-^{13}C HSQC	2	512	39 min
2-D ^{1}H-^{13}C HMBC	2	512	40 min

units have been reported with the anomeric β-configuration, while the arabinosyl and rhamnosyl units have the α-configuration. Be aware that although the D- and L-form of many anthocyanin monosaccharides have been published, these assignments are lacking experimental verification.

The single ^{1}H-^{13}C bond of the aglycone and monosaccharides of selected anthocyanins have been found to be between 125 and 175 Hz (Pedersen et al., 1995).

Time Considerations

The time required for each type of NMR experiment depends on many factors, including sample concentration and complexity, shimming, magnetic field strength, and the sensitivity of the individual experiments. Indication of time requirements of individual 1-D and 2-D experiments are given in the legends of Figures F.1.4.2, F.1.4.3, F.1.4.5, F.1.4.6, and F.1.4.7, as well as Table F1.4.9.

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APPENDIX

NMR Glossary

Chemical shift (δ): A dimensionless quantity defined as: $\delta = (\nu_{sample} - \nu_{reference})/\nu_0 \times 10^6$, where ν_{sample} is the resonance frequency of the sample, $\nu_{reference}$ is the resonance frequency of the reference, tetramethylsilane (TMS; defined as zero), and ν_0 is the observing frequency (e.g., 300 or 600 MHz). The unit for the δ scale is ppm (parts per million) and is independent of the strength of the applied magnetic field. Exact resonance frequency of a nucleus is a function of the environment (chemical/magnetic) of the observed nuclei.

Coupling constant (J in Hz): Interaction between nuclear spins mediated through chemical bonds giving rise to mutual splitting of resonance lines.

Free induction decay (FID): An oscillating voltage recorded as the magnetization vector precesses (rotates) in the laboratory frame.

Fourier transformation (FT): Mathematical operation to convert a time domain spectrum (FID) to a frequency domain spectrum (normal NMR spectrum).

Memory locations (number of data points): Number of data points stored. Proportional to the acquisition time.

Relaxation: Energy loss to the surroundings of an excited nucleus when it returns to its ground state. (1) Spin-lattice relaxation in which the relaxation process involves the entire framework or aggregate of neighbors of the high-energy nucleus. (2) Spin-spin relaxation which implies transferring the excess energy, ΔE, to a neighboring nucleus, provided that the particular value of ΔE is common to both nuclei.

Scan: Individual FID. Usually the intensity of an individual FID is so weak that the FIDs of many pulses are added together (accumulated) in the computer unit to obtain a stronger signal—i.e., better signal-to-noise ratio (S/N)—before the signal is transformed.

Signal-to-noise ratio (S/N): Increases in proportion to the square root of the number of scans (ns). The strength of the NMR signals are thus improved by increasing the number of scans.

Spectral width or sweep width (SW): The defined range of frequencies in which signals are expected to be found.

Extraction, Isolation, and Purification of Carotenoids

Recently, food processors and technologists have shown a great interest in the isolation, identification, and purification of natural pigments, including carotenoids, due to their nutritional value, their use as colorants, and their potential as health aids. This unit describes a practical way of extracting, isolating, and purifying carotenoids from plant materials. The method is based mainly on the natural form in which the carotenoids are found (esterified or free) and to some extent on their polarity and/or solubility in the solvents used. Common and readily available solvents and laboratory equipment are suggested.

The extraction and isolation of three groups of carotenoids of different polarity are described in Basic Protocol 1. A method for prepurifying carotenoids using crystallization is described in Basic Protocol 2. Carotenoids may be purified further by chromatographic techniques (*UNIT F2.3*) and characterized (*UNITS F2.2 & F2.4*). Support Protocols 1 and 2 describe the preparation of the sample before extraction. This process consists mainly of removing water from the sample followed by sample grinding or homogenizing.

NOTE: Carotenoid pigments should be protected from light, oxygen, and heat (see Critical Parameters and Troubleshooting).

SOLVENT EXTRACTION AND ISOLATION OF CAROTENOIDS

BASIC PROTOCOL 1

This protocol begins with the extraction of a dehydrated sample. It continues with a saponification scheme to initiate the isolation of the carotenoid mixture. During saponification, the esters are hydrolyzed and the free pigments released. Then, to continue the isolation, column chromatography is suggested as a simple and fast means of separating the three main groups of carotenoids based on their different polarities.

Materials

Dehydrated plant material (see Support Protocols 1 and 2)
Extractant: 1:1 (v/v) hexane/acetone or hexane alone
Saponifying solution: 40% (w/v) KOH in methanol (cool to room temperature before bringing up to volume)
Salting-out solution: 10% (w/v) Na_2SO_4
Na_2SO_4, anhydrous (powder form)
Adsorbent (see recipe)
Carotene eluant: 4% (v/v) acetone in hexane
Monohydroxy pigment (MHP) eluant: 1:9 (v/v) acetone/hexane
Dihydroxy pigment (DHP) eluant: 1:8 (v/v) acetone/hexane

500-ml extraction vessel
Explosion-proof shaft mixer (e.g. model SIU04X; Lightnin)
Whatman no. 42 filter paper
Rotary evaporator (e.g., Büchi Rotavapor, Brinkmann Instruments) attached to vacuum pump, ≤55°C
56°C water bath
125-ml separatory funnel
600 × 40–mm chromatography column
Glass wool
Vacuum filtration device
1-liter filtration flasks

Contributed by Gustavo A. Rodriguez
Current Protocols in Food Analytical Chemistry (2001) F2.1.1-F2.1.8

Extract carotenoids

1. Place dehydrated plant material (and filter paper if Support Protocol 2 was used) in a 500-ml extraction vessel.
2. Add 3 vol (v/w) extractant and mix 15 min using an explosion-proof shaft mixer to suspend paste in solvent.

   The type of extractant should be chosen based on the carotenoids of interest (see Commentary).

   No heating is necessary.
3. Vacuum filter mixture using Whatman no. 42 filter paper. Save filtrate.
4. Separate filtrant cleanly from filter paper, if possible, and extract a second time using 2 vol extractant. Continue extractions using smaller volumes of extractant until no appreciable color is observed in filtrate. Save and pool all filtrates.

   If filtrant cannot be separated from paper, both should be placed in the extraction vessel.

   At this point, concentrated filtrate from the sample preparation (see Support Protocol 2, step 8) may be pooled with the filtrates.
5. Concentrate extracts to ~40 ml in a rotary evaporator attached to a vacuum pump at ≤55°C.
6. Add 3 ml saponifying solution and stir 45 min at 56°C.

   This is the temperature recommended for hot saponification of pigments by the AOAC (1990).
7. Transfer saponified extract to a 125-ml separatory funnel and add 1 vol salting-out solution.
8. Remove bottom layer and wash upper layer three times with 10 ml water.
9. Add 3 g anhydrous Na_2SO_4 and filter using Whatman no. 42 filter paper. Save filtrate.

Isolate carotenoids

10. Plug the bottom of a 600 × 40–mm chromatography column with glass wool. Mount the column on a vacuum filtration device, using a 1-liter filtration flask as a receiving vessel (Figure F2.1.1).
11. Add adsorbent to obtain a 20-cm layer while applying vacuum.
12. Level the surface of the adsorbent and place a firm 2-cm layer of anhydrous Na_2SO_4 on top.
13. Pour carotene eluant into column until eluant wets all of the adsorbent.
14. Replace receiving vessel with a clean flask and pour filtrate (step 9) into column.
15. Allow all the sample to penetrate into the adsorbent and then add carotene eluant until the first carotenoid band that separates is completely collected in the flask.

    The carotenes and esters present in the sample are contained in this fraction. Other carotenoids remain on the column.

    The chromatography process is monitored visually. The exact volume of eluant added to the column will vary depending on sample concentration and composition.
16. Elute monohydroxy pigments with MHP eluant and dihydroxy pigments and more polar pigments with DHP eluant, using a clean receiving flask for each. If necessary, store pigments ≤24 hr at 0° to 5°C and protect from light.

    Eluates should be processed as soon as possible.

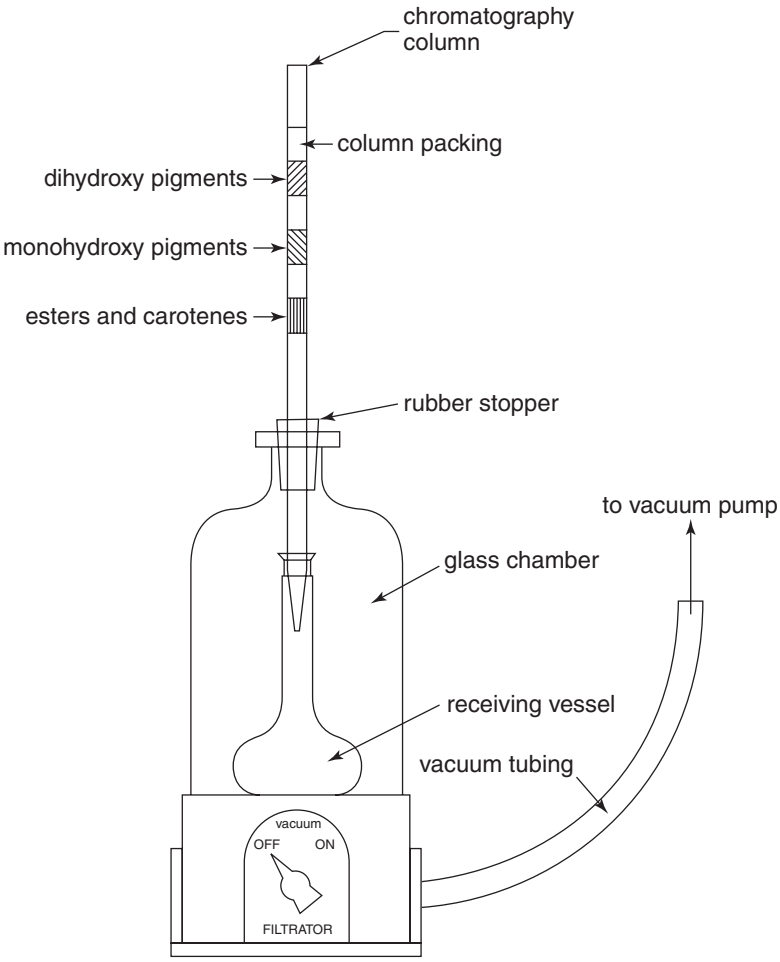

Figure F2.1.1 Rapid separation and collection of pigments using column chromatography with vacuum filtration.

PREPURIFICATION OF CAROTENOIDS BY CRYSTALLIZATION

BASIC PROTOCOL 2

Once the carotenoids have been isolated as described in Basic Protocol 1, they can generally be crystallized as an initial step to purification. Actually, what is most likely to happen is a co-crystallization. When working with a nonpolar fraction, α- and β-carotene may co-crystallize. In the same way, a polar fraction may yield lutein-zeaxanthin crystals. A pure carotenoid product may be obtained by crystallization of a fraction derived from a preparatory chromatographic procedure, which can be done using TLC, HPLC (*UNIT F2.3*), or in some cases column chromatography.

Materials

Isolated carotenoid eluate (see Basic Protocol 1, step 15 or 16)

Precipitating solvent: 1:1 (v/v) hexane/acetone or hexane alone, room temperature and cold (0° to 5°C)

50- or 125-ml pear-shaped flasks

Rotary evaporator (e.g., Büchi Rotavapor, Brinkmann Instruments) attached to vacuum pump.

Nitrogen gas tank with regulator adaptable to rotary evaporator and vacuum oven

Whatman no. 42 filter paper

Vacuum oven, 40°C

1. Place isolated carotenoid eluate in a 50- or 125-ml pear-shaped flask and concentrate eluate to near saturation in a rotary evaporator attached to a vacuum pump under a stream of nitrogen from a nitrogen gas tank.

   Only one carotenoid eluate (carotenes, MHPs, or DHPs) should be prepurified at a time.

   The flask size will depend on the volume of eluate to be concentrated.

2. Add precipitating solvent drop by drop until the solution starts turning cloudy, indicating that precipitation has begun.

   A solvent mixture should be selected for crystallization that will easily solubilize the carotenoid of interest and also, when added in small quantities, will force the carotenoid out of solution and start crystallization. The extractants from Basic Protocol 1 (hexane/acetone or hexane alone) are appropriate.

3. Refrigerate (0° to 5°C) solution overnight.

   Crystallization can be accelerated by placing solution at −20° to 0°C for 3 or 4 hr. Usually better crystals are produced by a slow crystallization.

4. Vacuum filter crystals using Whatman no. 42 filter paper. Wash crystals with cold (0° to 5°C) precipitating solvent.

5. *Optional:* Repeat crystallization sequence to obtain crystals of higher purity, beginning with a saturated solution of crystals (step 4) in acetone and continuing with step 2.

6. Dry crystals in a vacuum oven at 40°C, flushing drying chamber occasionally with nitrogen.

7. Pack crystals under nitrogen and store ≤6 months at −20°C if further purification work is anticipated.

SUPPORT PROTOCOL 1

REMOVAL OF WATER FROM CAROTENOID-CONTAINING SAMPLES USING A VACUUM OVEN

Plant tissues contain variable amounts of water, which is easily eliminated in order to facilitate extraction with organic solvents. Using a low-temperature vacuum oven is a good way of removing water from materials that are easily pulverized. This is true for many vegetables.

Materials

Plant tissue of interest
Vacuum oven, 60°C
Laboratory mill with 30-mesh screen

1. Weigh plant tissue of interest and record weight.

   The initial wet weight is important as, in many cases, extraction and purification of carotenoids are done to quantify pigment concentrations, which must be related to the fresh material.

   The amount of tissue used is dependent on the expected pigment concentration. Usually 100 to 500 g is appropriate.

2. Place sample inside a vacuum oven at 60°C. Maintain set temperature.

3. Turn on and control the vacuum between 12 and 20 in. Hg (305 to 508 mmHg).

4. Open oven door occasionally during drying and stir material to obtain uniform dehydration.
5. Dry to ~8% moisture content.

   Overdrying can damage the carotenoids present in the sample.

   A moisture balance calibrated to indicate an 8% moisture content can be used to monitor weight loss and may be useful until experience is gained. The calibration can be performed as a gravimetric determination on a sample analyzed in parallel. This measurement can also be used to estimate an 8% moisture content relative to the initial sample weight.
6. Grind material to 30 mesh with a laboratory mill.
7. Weigh powder for extraction.

   The weight will also be indicative of residual moisture if the initial amount is known (see Chapter A1).

REMOVAL OF WATER FROM CAROTENOID-CONTAINING SAMPLES USING ALCOHOL

SUPPORT PROTOCOL 2

This is a practical way of removing water from plant tissues when water is needed for homogenization or when the dry material cannot be ground to a powder due to the presence of lipids, waxes, or sugars.

Materials

Plant tissue of interest
95% (v/v) ethanol
Hexane

Explosion-proof shaft mixer (e.g., model SIU04X; Lightnin)
Whatman no. 41 filter paper
Separatory funnel
Rotary evaporator (e.g., Büchi Rotavapor, Brinkmann Instruments) attached to vacuum pump, ≤55°C

1. Weigh plant tissue of interest as described (see Support Protocol 1, step 1).
2. Mix plant tissue to a paste with an explosion-proof shaft mixer. If the material does not lend itself to mixing, add sufficient water to homogenize sample.
3. Add 2 vol of 95% ethanol to homogenate. Mix thoroughly 5 min.
4. Vacuum filter homogenized sample using Whatman no. 41 filter paper. Save filtrate.
5. If filtrate is cloudy, repeat steps 3 and 4 using 1 vol of 95% ethanol. If filtrate is clear, continue with step 6.

   A cloudy filtrate is an indication that additional alcohol is needed.
6. Remove and save filtrant along with the filter paper. Store ≤24 hr at −20°C.

   The sample and the filter paper are now ready for extraction.
7. Pool filtrates in a separatory funnel. Add 1 vol hexane.

   Some of the more polar pigments are usually carried into the filtrate and must be recovered by extraction.
8. Remove bottom layer and concentrate upper layer containing the lipophilic carotenoids in a rotary evaporator attached to a vacuum pump at ≤55°C. Store ≤24 hr at −20°C.

REAGENTS AND SOLUTIONS

All solvents used must be good grade analytical reagents and water should be distilled. For suppliers, see ***SUPPLIERS APPENDIX****.*

Adsorbent

Mix equivalent weights of silica gel 60 GF_{254} (Merck) and diatomaceous earth (Hyflo Super-Cel; Celite, World Minerals) in a mechanical blender or in a large plastic bag for 2 hr. Store ≤3 months in a sealed container at room temperature.

COMMENTARY

Background Information

Carotenoids have been of great interest, and their importance in food coloration has been well reviewed by Kläui and Bauernfeind (1981). Extraction, isolation, and purification are described excellently by Schiedt and Liaaen-Jensen (1995).

This unit focuses on procedures for obtaining the important carotenoids that are found naturally in foods and plant material and that are of nutritional and pharmacological interest. The pigments referred to include α- and β-carotenes, β-cryptoxanthin, lutein, zeaxanthin, lycopene, capsanthin, and capsorubin, among others.

For extraction, water content is considered an important factor. It has been found that working with low-moisture samples simplifies the extraction process. Industrial extraction normally is done with dry material, which reduces complications arising from the solvents used for processing and their recovery.

The plant material as prepared in Support Protocols 1 and 2 is ready for extraction. It may contain some water (≤10%), which will not affect extraction. Freeze-drying may be another way of eliminating water with little pigment damage and may be an acceptable alternative to drying in a vacuum oven as long as the moisture content is taken to an appropriate level for efficient extraction. Freeze-drying is not adequate if sugar or other water-soluble compounds must be eliminated by physical separation (e.g., by filtration or centrifugation).

The solvent used for extraction must be chosen according to the polarity of the pigments presumably present. If this characteristic is unknown, an acetone/hexane (1:1, v/v) mixture is suitable. When it is known that the carotenoids in the sample are nonpolar or are in the ester form, hexane is a good choice for extraction. Ethanol will extract polar carotenoids, and a nonpolar solvent like hexane will promote crystallization.

In this unit, extraction is carried out using solvents. Other methods for extracting pigments are available. The use of supercritical carbon dioxide is described by Favati et al. (1988), Chao et al. (1991), and Spanos et al. (1993). Enzymes may also be used to help digest plant material and then facilitate pigment extraction by physical or chemical means (Sims et al., 1993; Thomas et al., 1998; Koch et al., 1999). Ultrasound may also be used to aid the extraction process; it is used in laboratory and industrial work for extracting pigments and other compounds. Vegetable oil may be used as an extractant and pigment carrier. However, using oils for extraction produces low pigment concentrates. By introducing organic solvents, pigments are more easily solubilized, and extracts of high purity may be obtained.

Following extraction, an efficient way of initiating the isolation of carotenoids is to saponify the extract. This removes many of the unwanted lipids present in the sample as well as chlorophyll. The saponification by-products, which to a great extent are sodium or potassium salts, are easily separated by an aqueous solution of a highly polar salt. The addition of water also helps wash off excess alkali and other water-soluble and water-complexed compounds. This procedure hydrolyzes xanthophyll esters to form the hydroxylated carotenoid.

After saponification, column chromatography is applied as recommended in the Official Methods of Analysis of the AOAC (1990), although a larger column is used to accommodate the larger sample volume. This type of chromatography is very practical for most laboratories and is very much in use in those that specialize in carotenoid studies. Efficient separation of the main groups of carotenoids is achieved using solvents of different polarity. In this standard analytical procedure, the first eluant that is put through the column is a nonpolar solvent, such as hexane or a 90:10 (v/v) hexane/acetone mixture, to elute carotenes and xanthophyll esters

(all low-polarity compounds). An 80:20 (v/v) hexane/acetone solvent combination may be used to elute moderately polar monohydroxy carotenoids, and a 60:40 (v/v) hexane/acetone mixture may be used to elute the very polar fraction of dihydroxy pigments. The recommended solvents in Basic Protocols 1 and 2 are the ones found to be the most convenient for these purposes.

Crystallization as described in Basic Protocol 2 generally provides products with two or more pigments, but sometimes produces crystals of a single carotenoid.

Critical Parameters and Troubleshooting

When working with carotenoid pigments, it is difficult to overemphasize the need to protect these compounds from light, oxygen, and heat. It is therefore necessary to work in dim light and to keep intermediary products in the dark. Also, wherever possible, a vacuum or inert atmosphere must be used. High temperatures should be avoided, and samples being processed should be kept in a refrigerator or freezer when work is discontinued.

Complete dehydration before extraction is not recommended. A small amount of water is often useful when a low-polarity solvent mixture is the extractant. On the other hand, excess water may make extraction inefficient. Using the right extractant is of great importance and depends largely on which carotenoids are sought. Using the hexane/acetone mixture as the extractant is advantageous because the same pair of solvents is used later for crystallization.

Once carotenoids have been extracted and isolated, they tend to be very unstable, as natural antioxidants and protecting agents have been stripped away. An antioxidant (such as BHT, a tocopherol blend or vitamin E, rosemary extract, or other food-grade chemical) should then be added to the carotenoid. Many time-encapsulating agents or other substances are added to the carotenoid concentrates, but the antioxidants may represent ~5% of the total formulation (Vilstrup et al., 1998; Kowalski et al., 2000). The presence of metals and chlorophylls will also affect pigment stability, but this problem is largely solved in the salting-out step of Basic Protocol 1.

The salting-out may produce an interface that often has a high pigment concentration and occasionally even crystals that begin to precipitate. It is important to collect the interface and wash it with salt solution or an appropriate solvent to allow the pigments to combine with the upper phase. This interface may also contain hydrated phospholipids or some other waxy material that can be reduced or eliminated by filtration, followed by washing of the filter cake with the extractant to recover pigment. Often the use of an inert filter aid will speed up filtration and will also capture the undesirable matter.

When the sample has been passed through the chromatography column in Basic Protocol 1, the isolates are in a solvent mixture (hexane/acetone) that can be used for crystallization after saturating by evaporation and enhancing precipitation. If a different solvent pair is necessary for crystallization, the hexane/acetone may be eliminated in a rotary evaporator and the new solvent pair may be added to the concentrate.

Anticipated Results

Yields in milligrams of the pigment sought are fully dependent on the content in the raw sample. Nevertheless, these protocols typically produce isolates in the range of 50% to 70% purity. After prepurification by a single crystallization step, purity of the concentrates will range between 65% and 85%. It should be pointed out that the pure crystalline forms are normally not obtained. As mentioned above, preparative chromatography (*UNIT F2.3*) will produce fractions that can be crystallized or simply dried to a pure form.

Time Considerations

Completion of the full process will take 3 to 4 days depending on the dehydration protocol chosen. Vacuum drying (Support Protocol 1) will take a full day. Extraction, isolation, and startup of prepurification will take another day. It is advisable to crystallize overnight and to filter, wash, and dry the crystals the next day. If a second crystallization step is desirable add another day.

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Technical advancements in carotenoids are followed up from the original edition edited by Otto Isler.

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Detection and Measurement of Carotenoids by UV/VIS Spectrophotometry

The majority of carotenoids exhibit absorption in the visible region of the spectrum, between 400 and 500 nm. Because they obey the Beer-Lambert law (i.e., absorbance is linearly proportional to the concentration), absorbance measurements can be used to quantify the concentration of a pure (standard) carotenoid (see Basic Protocol 1) or to estimate the total carotenoid concentration in a mixture or extract of carotenoids in a sample (see Basic Protocol 2). Considerations for the preparation of carotenoid-containing samples are presented in Critical Parameters (see Sampling and Sample Preparation).

NOTE: Carotenoids are easily degraded. See Critical Parameters for a discussion of suitable precautions.

NOTE: All extinction coefficients in this unit assume a 1-cm pathlength.

PREPARATION AND CALIBRATION OF INDIVIDUAL CAROTENOID STANDARDS

BASIC PROTOCOL 1

In this protocol, commercially purchased carotenoid standards are dissolved in a suitable solvent and the absorbance measured at its maximum wavelength (λ_{max}). Using published extinction coefficients and taking into consideration the dilution factor, the concentration of the standard carotenoid is calculated. The spectrum is also scanned in order to evaluate the fine structure (see Spectral Fine Structure in Background Information). The carotenoid solution should ideally be assayed by HPLC as described in *UNIT F2.3* to establish chromatographic purity and thus correct the calculated concentration.

Materials

~1 to 5 mg standard carotenoids (Table F2.2.1 and Table F2.2.2)
High-grade organic solvent (Table F2.2.2)
10- to 50-ml volumetric flask
Additional reagents and equipment for HPLC analysis (*UNIT F2.3*)

1. Dissolve carotenoid (e.g., ~1 to 5 mg) in a suitable solvent (see Table F2.2.2). Make to an accurate volume (e.g., 10 to 50 ml) in a volumetric flask.

   A larger volume may be used if desired.

Table F2.2.1 Commercial Sources of Carotenoids[a]

	Sigma Aldrich	Extra-synthese	Atomergic Chemical	Indifine Chemical	Fisher Scientific	Fluka Chemical	Carl Roth GmgH
β-carotene	X	X	X	X	X	X	X
α-carotene[b]							
Lycopene	X	X	X	X			X
β-crytoxanthin	X	X	X			X	
Zeaxanthin		X	X	X			X
Lutein	X	X	X	X			X

[a]The companies may have offices or distributors in other countries.
[b]At the time of writing there were no commercial suppliers of α-carotene.

Contributed by K. John Scott
Current Protocols in Food Analytical Chemistry (2001) F2.2.1-F2.2.10

It is essential that the carotenoid be completely dissolved. With crystalline samples, dissolution can be aided by initial addition of a small amount of a more effective solvent (e.g., dichloromethane) prior to making to volume for spectrophotometric measurement (for most commonly assayed carotenoids, no more than 10% of the total volume should be required; however, lycopene may require up to 100%). Where a carotenoid is supplied in a sealed ampule, dissolution can be conveniently achieved by adding successive small aliquots of the more effective solvent to the ampule, and transferring to a volumetric flask. It is not easy to assess complete dissolution visually; therefore, it is advisable to filter the solution through a suitable solvent-compatible 0.45-μm filter.

2. Dilute the solution (e.g., 1:50) in desired solvent if necessary to give ~0.3 to 0.7 AU as measured on a spectrophotometer at λ_{max}.

   It is recommended that at least two independent dilutions be made to ensure confidence in the measurement.

   If a larger final volume is used (see step 1) reduce the dilution accordingly.

3. Warm up the spectrophotometer per manufacturer's instructions.

4. Zero the spectrophotometer with solvent in a cuvette.

   NOTE: *Cuvettes must be kept scrupulously clean; avoid handling the surfaces of the cell (see Critical Parameters).*

5. Place a cuvette containing the carotenoid solution into the sample cell holder of the spectrophotometer.

6. Measure the absorbance at λ_{max}. Take reading immediately.

   See Critical Parameters concerning degradation.

7. Scan to allow measurement of the fine structure (see Table F2.2.3; also see Spectral Fine Structure in Background Information).

   The same principles can be applied to measurement of chromatographic fractions.

8. Calculate the concentration of carotenoid as shown in the example below for all *trans*-β-carotene.

$$\frac{A \times V_1}{A^{1\%}} \times C^{1\%} = \frac{0.5 \text{ AU} \times 50}{2592 \text{ AU}} \times 10 \text{ mg/ml} = 96.5 \text{ μg/ml}$$

Table F2.2.2 Data on λ_{max} and Extinction Coefficients of a Selection of Carotenoids

	MW	$A^{1\%}$	$\varepsilon^{1 \text{ mM}}$	λ(nm)	Solvent	%III/II
α-carotene	537	2710	145	445	Hexane	55
β-carotene	537	2592	139	450	Hexane	25
β-cryptoxanthin	553	2460	136	450	Hexane	25
Lutein	569	2550	145	445	Ethanol	60
Lycopene	537	3450	185	470	Hexane	65
Zeaxanthin	569	2480	141	450	Hexane	25
Natural carotenoids as food colors						
Bixin (Bixa orellana)	395	4200	166	456	Petroleum ether	
Capsanthin (paprika)	585	2072	121	483	Benzene	
Capsorubin (paprika)	601	2200	132	489	Benzene	
Synthetic food colors						
β-apo-8′-carotenal	417	2640	110	457	Petroleum ether	
Canthaxanthin	564	2200	124	466	Petroleum ether	0

Where A is the absorbance reading of the diluted sample (0.5 AU), V_1 is the dilution factor (50×), $A^{1\%}$ is the absorbance of a 1% solution (i.e., the extinction coefficient; 2592 AU), and $C^{1\%}$ is the concentration of a 1% solution (10 mg/ml). Using this formula, the concentration of the original solution in this example is = 96.5 μg/ml.

9. Subsequent to measurement of the carotenoid concentration, the solution should be assayed by HPLC to establish the chromatographic purity (see *UNIT F2.3*).

   For example, assuming the same values as Equation F2.2.1, if the total chromatographic area is 10000, and the area of all-trans β-carotene peak is 9500, then the chromatographic purity is 9500/10000 × 100 or 95%, and the actual concentration of β-carotene is 95.5 μg/ml × 95/100 or 91.7 μg/ml.

MEASUREMENT OF TOTAL CAROTENOID CONCENTRATION IN FOOD COLORANTS, PHARMACEUTICALS, AND NATURAL EXTRACTS

BASIC PROTOCOL 2

The same principle as described above can be used for the "estimation" of the carotenoid content of extracts of food colorants, pharmaceuticals, foods, biological samples, or chromatographic fractions. This procedure employs calculations used for individual carotenoids of high purity and thus will estimate the "total carotenoids" present in a food or biological extract, where a mixture of carotenoids would be expected. Greater accuracy can be obtained as extracts are purified to contain single components (see Commentary). A spectrum scan is not employed in this procedure as the fine structure of a mix of carotenoids can only be identified after HPLC separation (see Commentary).

Materials

Sample
Appropriate solvent (Table F2.2.2)
Additional equipment and reagents for sample extraction (*UNIT F2.1*).

1. Prepare the sample as detailed in *UNIT F2.1*, taking into consideration the guidelines detailed in Critical Parameters (see Sampling and Sample Preparation). Dilute the sample appropriately using a suitable solvent.

   Samples containing esterified carotenoids, chlorophyll, or high levels of fat may require saponification (UNIT F2.1).

2. Warm up spectrophotometer per manufacturer's instructions.

3. Zero the spectrophotometer with solvent in a cuvette.

Table F2.2.3 Additional Spectral Characteristics of Carotenoids

Common name	Chemical name	Solvent	Absorption peaks		
β-carotene	(β,β-carotene)	Hexane	425	450	478
α-carotene	(β,ε-carotene)	Hexane	422	445	473
Lycopene	(φ,φ-carotene)	Hexane	444	470	502
β-cryptoxanthin	(3-hydroxy-β-carotene)	Hexane	428	450	478
Zeaxanthin	(β,β-carotene-3,3′-diol)	Hexane	425	450	478
Lutein	(β,ε-carotene-3,3′-diol)	Ethanol	421	445	474
Capsanthin		Petroleum ether	450	475	505
Capsorubin		Petroleum ether	445	479	510
Bixin		Petroleum ether	432	456	490
Canthaxanthin		Petroleum ether		466	
β-apo-8′-carotenal		Ethanol	405	430	460

4. Place a cuvette containing a suitably diluted (i.e., ~0.3 to 0.7 AU) extract of pharmaceutical, food, or biological material into the sample cell holder of the spectrophotometer.

5. Measure absorbance at selected λ_{max} (Table F2.2.2 and Table F2.2.3). Read immediately.

   See Critical Parameters concerning degradation.

6. Estimate the total carotenoid concentration of the sample (see Basic Protocol 1, step 9 and Equation F2.2.1).

   With the exception of individual food colorants and single carotenoid pharmaceutical products, it is unlikely that the extract will be composed of only one predominant carotenoid; therefore, a specific λ_{max} or extinction coefficient (Table F2.2.2) cannot be used. In this case it is convenient to use a λ_{max} of 450 nm and a typical $A^{1\%}$ value of 2500. Alternative values and other considerations are discussed elsewhere (see Commentary).

COMMENTARY

Background Information

The majority of carotenoids exhibit absorption in the visible region of the spectrum, mainly between 400 and 500 nm (see Table F2.2.2 for examples). A few carotenoids (e.g., phytoene) exhibit maximum absorbance in the UV region. This absorption is due to the long conjugated double bond system of carotenoids. The conjugated unsaturated part of the carotenoid molecule containing delocalized π-electrons is called the "chromophore" and is re-

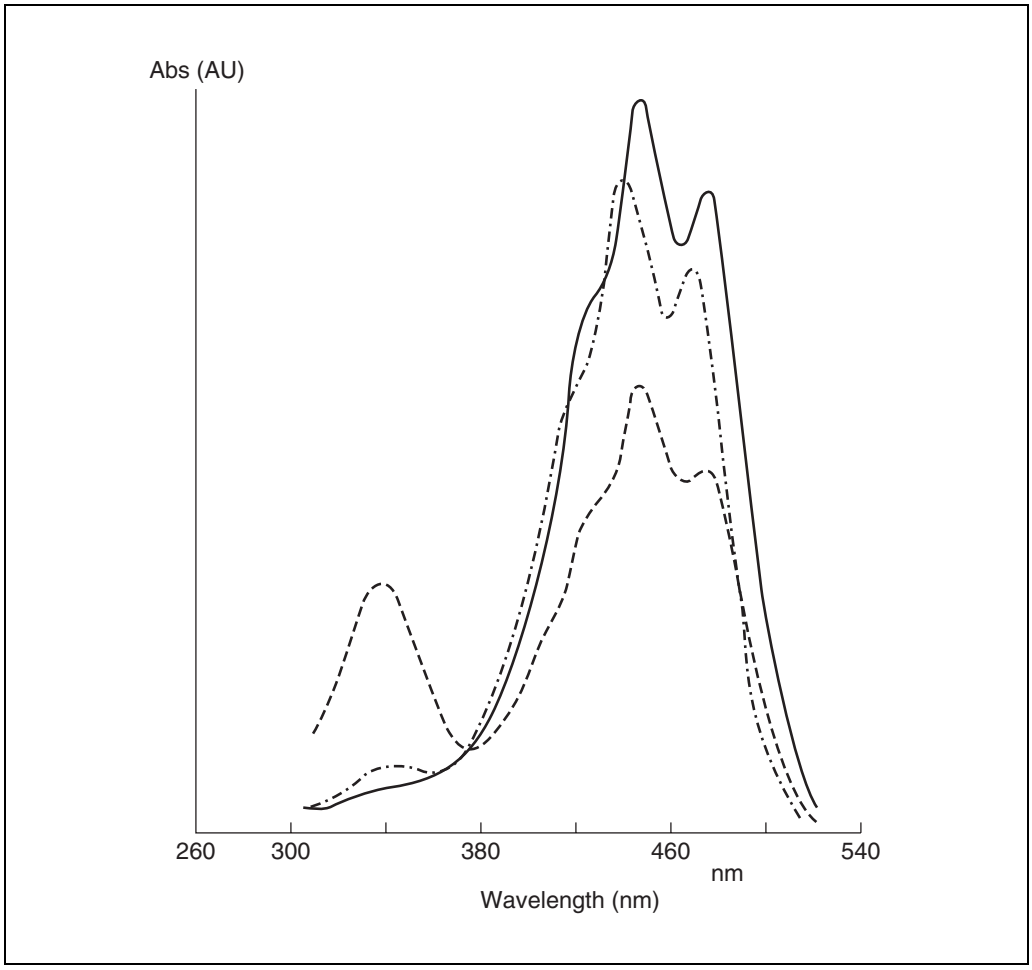

Figure F2.2.1 The spectral characteristics of *all-trans* β-carotene (solid line), 9-*cis* β-carotene (dashed and dotted line) and 15-*cis* β-carotene (dashed line).

sponsible for the absorption of light in the visible region.

The differences in the spectral characteristics of individual carotenoids are often small, but are of great importance in their identification; however, carotenoids having the same chromophore, such as β-carotene and its hydroxy-derivative zeaxanthin, have identical spectra. The spectra of *cis*- or *Z*-isomers of carotenoids, while being similar to the *all-trans* or *all-E* form, are different in as far as they exhibit a change in the λ_{max} to a shorter wavelength, a decrease in the magnitude of the absorbance, a reduction in the fine structure, and additional absorption bands in the UV region around 340 nm and 280 nm (Fig. F2.2.1). It is not the intention of the author to go into any great detail of the molecular characteristics of carotenoids in this unit, as details can be found in a excellent chapter by Britton (1995).

Extinction coefficients

Carotenoids in solution obey the Beer-Lambert law, where absorbance (*A*) equals concentration multiplied by extinction coefficient ($A^{1\%}$), where the extinction coefficient ($A^{1\%}$) is defined as the absorbance of a 1% (10 g/liter) solution of carotenoid, in a defined solvent, in a 1-cm path-length cuvette, at a specific wavelength (λ). This information can be used to quantify the concentration of a pure (standard) carotenoid (see Basic Protocol 1), or to "estimate" the total carotenoid concentration in a mixture or extract of carotenoids in a sample (see Basic Protocol 2). The extinction coefficient may also be expressed in terms of molarity.

As seen in Table F2.2.2, a 1% solution (10 g/liter) of β-carotene has an absorbance of 2592 AU and a molecular weight of 537 g/mol (i.e., 1 mM = 0.537 g/liter); therefore, the absor-

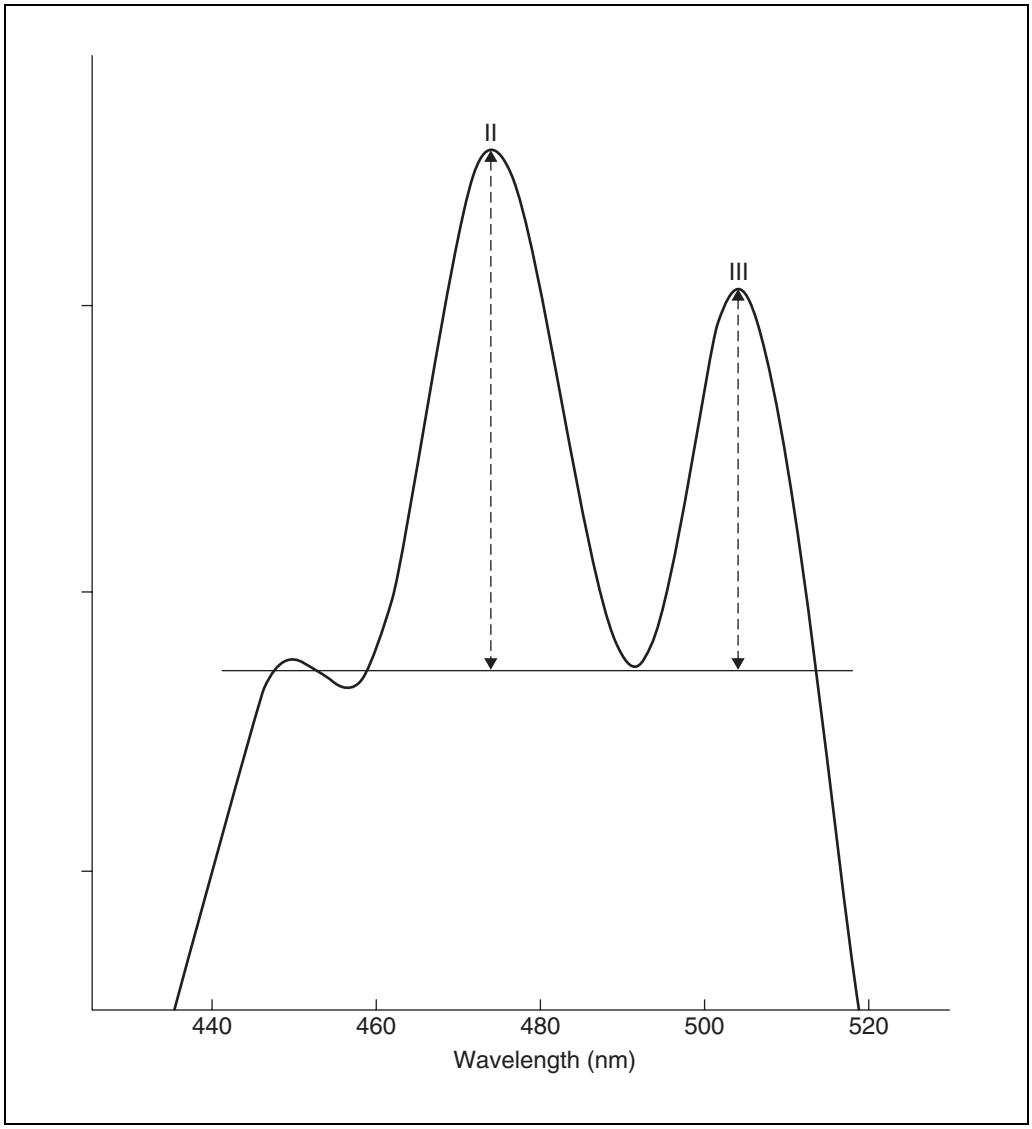

Figure F2.2.2 The calculation of %III/II from the spectral fine structure.

bance expected for a 1 mM solution is: (0.537 g/liter)/(10 g/liter) × (2592 AU) = 139 AU/1 mM β-carotene. Using this ratio, if a β-carotene solution has an absorbance of 5, then the concentration is given as: (5 AU)/(139 AU/mM) = 0.036 mM. It is important to note that due to the inherent difficulties in procedures for accurate determination of extinction coefficients, there may be a significant level of uncertainty in some published values. Small variations (e.g., 2 to 3 nm) may also occur in published data of absorption maxima. Whenever possible, the spectrum of a compound under investigation should be compared directly with an authentic pure standard. The spectra of the unknown and the standard should be identical for both the λ_{max} and the fine structure (see below).

Spectral fine structure

In addition to the absorption maxima of the carotenoids, the "shape" of the spectra provides important information for identification of purified carotenoid extracts or pure standard (while the identity of the standard is generally not in question, it is a good idea to check the purity by fine structure analysis). Fine structure is demonstrated in Figure 2.2.2, and is measured as a ratio of the absorbance maxima to one of the shoulders (i.e., %III/II). The longest wavelength band is called III and the middle absorption band II. The baseline is taken as the minimum between the two peaks, and the height of each peak is measured. Carotenoids having the same chromophore, such as β-carotene and its hydroxy-derivative zeaxanthin, have identical fine structure, while conjugated ketocarotenoids, for example canthaxanthin, have only a rounded spectrum with no fine structure (see Fig. F2.2.3), thus the %III/II is 0.

Spectral monitoring during HPLC

The spectral characteristics of a standard can be monitored during HPLC using a diode-array detector (*UNIT F2.3*). A directory of standard spectra can be stored, enabling additional identification of sample peaks. The actual absorption maxima and fine structure will be dependent on the composition of the mobile phase (see Fig. F2.2.4). Peak I may only occur as a "shoulder" with *cis*-carotenoids, while an additional peak is observed at around 340 nm (see Fig. 2.2.1).

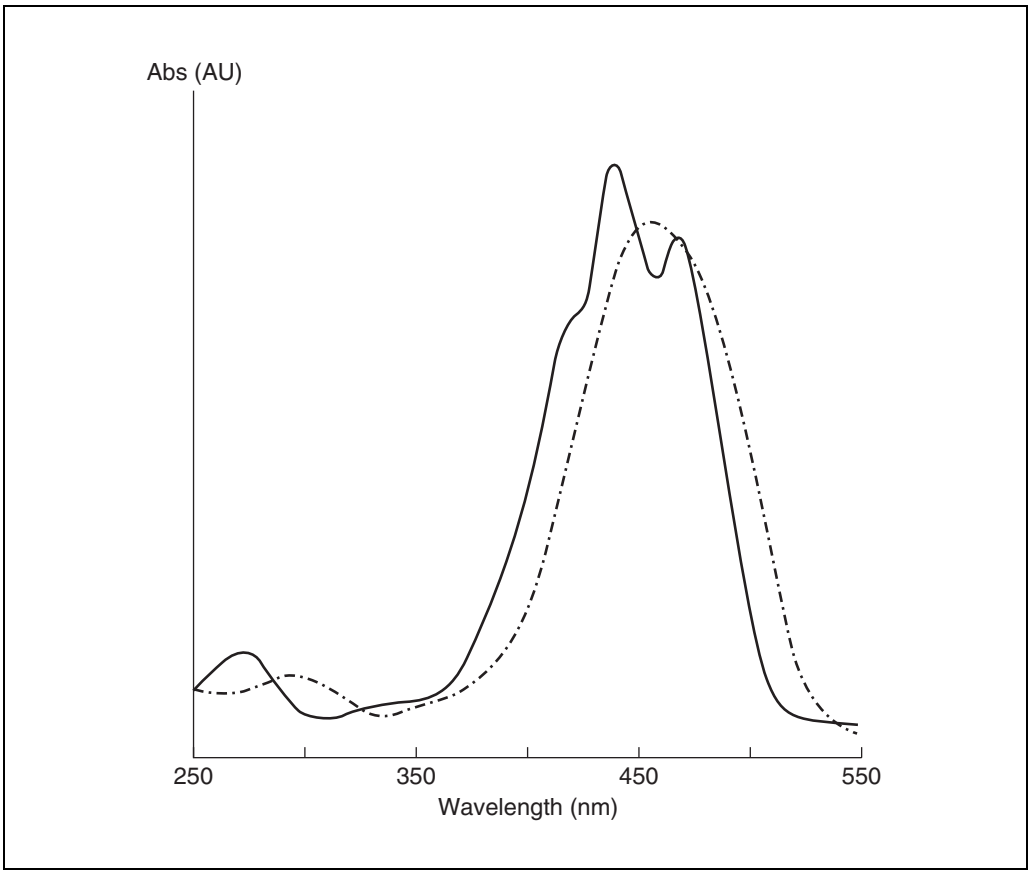

Figure F2.2.3 The spectral characteristics of β-carotene (solid line) and canthaxanthin (dashed line). Redrawn from original by Jaffé and Orchin (1962).

Critical Parameters and Troubleshooting

Good laboratory practice for UV/VIS spectrophotometry

While the procedures outlined above are fairly straight forward, in order to obtain maximum accuracy, "good laboratory practice" should be applied at all times.

1. Whenever possible the preparation and handling of carotenoid solutions should be carried out under yellow/gold fluorescent lighting to avoid light induced degradation.
2. All glassware should be scrupulously clean and reagents (e.g., solvents) should be of the highest quality.
3. The spectrophotometer should be located in a clean environment away from direct sunlight and drafts, at a reasonably even temperature, and free from electrical interference.
4. Any spillage should be cleaned up immediately.
5. When working with organic solvents it is advisable to use stoppered cells to prevent damage to cell holders and other sensitive parts of the instrument, and to avoid evaporation of the sample.
6. It is advisable to have the instrument regularly serviced (i.e., annually) by the manufacturer's engineer.
7. Once they have become familiar with the equipment, day to day maintenance can be carried out by the user; however, if in doubt, consult the manufacturer.
8. Modern equipment displays a range of error messages, often in the form of a letter and number, if the instrument fails or is not working to specification (e.g., failure of light source). Refer to the user's manual for details.
9. Cuvettes should be kept clean and the faces to be placed in the light beam should not be handled.
10. For normal measurement of carotenoids, glass cells can be used.

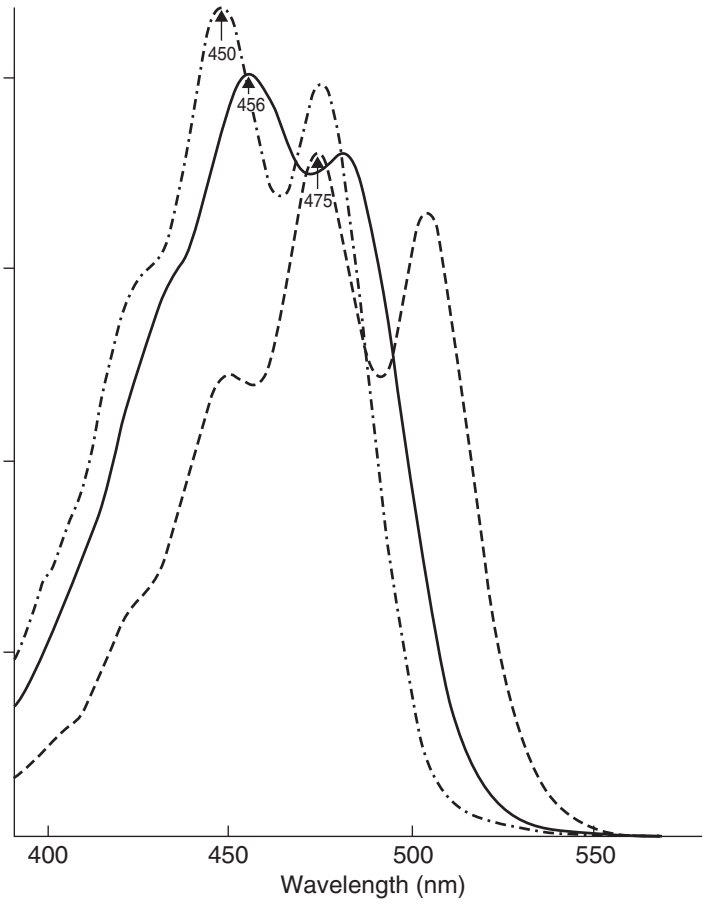

Figure F2.2.4 The spectral characteristics of β-carotene (solid line), lutein (long-dashed line), and lycopene (short-dashed line) in an acetonitrile-based HPLC solvent (75:25:5 v/v/v acetonitrile/methanol/dichloromethane).

11. It is not necessary to completely fill the cells, ~⅔ full is normally sufficient. This helps to avoid accidental spillage.
12. Before placing the cells in the cell holder, the optical faces can, if necessary, be polished carefully (avoiding any spillage) with a fine tissue.
13. For accurate results, it is advisable to use optically matched pairs or sets of cuvettes (i.e., cuvettes manufactured to a high specification to ensure they have the same optical parameters).
14. As pointed out above (see Basic Protocol 1), when preparing carotenoid standard solutions, complete dissolution of the solid material is essential; however, it is always advisable to filter the solution through a solvent compatible filter.
15. Stock solutions that have been stored (e.g., in a refrigerator), should be allowed to warm up to room temperature, refiltered, and a "new" concentration calculated prior to preparing a new working solution, which should be checked for chromatographic purity.
16. Spectrophotometric readings should be carried out immediately after the solution has been placed in the cuvette to avoid evaporation of the solvent or degradation.
17. Accurate measurement of standard solutions is a critical factor in the overall analysis of carotenoids. Inaccuracy of measurement can be a major cause of intra- and interlaboratory variation.
18. Experience will allow the analyst to recognize possible problems and anomalies, but a useful maxim is "if in doubt repeat it."

Sampling and sample preparation

Sample selection, number of samples, sample handling, and sample preparation prior to extraction are important factors effecting data quality. The type of sample (e.g., vitamin preparations, fruit drinks, supplemented foods, fruits and vegetables, biological materials), will determine to a large extent the sampling protocol and how the samples are handled. In this respect, the main considerations are the degree of homogeneity of the material and the possible variation in the vitamin content, not only between different materials, but also between different samples of the same material. Powdered, freeze-dried, and liquid materials for example, are likely to be more homogeneous with respect to vitamin distribution than fruits and vegetables. With fruits and vegetables, a good rule of thumb is that levels of vitamin in the outer part (e.g., outer leaves, skin, peel) are generally higher, considerably so in some cases, than the inner parts.

Equally with fruits and vegetables, the variety, origin, season of year, growing conditions, etc., will affect the vitamin content. In addition, consideration must be given to typical ways of preparation and cooking of the foods in the home. It is essential that the sampling protocol and the number of samples collected reflect the purpose of the exercise, be it to determine between batch variation of vitamin supplement preparations, the variation within vegetables of the same type, or to obtain a typical overall value for that vegetable. Ideally the time between sample collection and analysis should be as short as possible. The protocol should minimize any effects that may cause undesirable losses prior to analysis.

Frozen materials should be stored at −20°C, fruits and salad vegetables at around 4°C, and canned foods at room temperature. Powdered and freeze-dried materials should be stored in the dark in their original containers. Storage of fresh materials should preferably not exceed 3 days. After the initial preparation (see below), fresh or cooked materials can be conveniently stored at −20°C for a short time prior to extraction.

As indicated above, the preparation of the sample depends on the type of material and the homogeneity of that material. With dry powdered or liquid materials, the whole or parts of the samples collected can simply be thoroughly mixed prior to analysis.

Vegetables and fruits are prepared as appropriate for typical "in home" preparation methods (e.g., by removal of outside leaves, peeling, coring). Larger items such as cabbages, may be quartered, and then one quarter from each of the individual samples cut and mixed. Smaller items are cut and mixed. Further subsamples of the individual samples (e.g., 100 g depending on the number of individual samples collected initially) of the cut mixed materials are taken and these subsamples bulked and thoroughly mixed.

In order to obtain a thoroughly representative sample for extraction and subsequent analysis the following procedure has been used in the author's laboratory. Immediately after the preparation of the composite sample (raw and or after cooking as appropriate) the sample was frozen in liquid nitrogen and ground under liquid nitrogen in a Waring-type blender. Ground sample was then stored in an air-tight bottle under nitrogen at −20°C for up to 3 days

prior to analysis. All manipulations were carried out under yellow/gold fluorescent lighting.

Extraction

Methods of extraction are dealt with in detail in *UNIT F2.1*. All manipulations should be carried out under gold/yellow light, avoiding exposure to daylight or artificial "white" fluorescent light. All solvents must be of a high degree of purity. Cooking procedures such as boiling may cause disruption of the cellular matrix of vegetable material making the carotenoids more readily extractable. Samples containing high levels of fat, esterified carotenoids, or chlorophylls require saponification (see *UNIT F2.1*); however, in many instances chlorophylls may be separated from the carotenoids of interest during column chromatography avoiding the need for saponification. Certain saponification conditions may cause degradation of carotenoids, particularly the xanthophylls, so the concentration of KOH, time, and temperature must be carefully assessed for the particular type of material being analyzed.

Solvents

Different solvents and the composition of the mobile phase used in HPLC may effect lmax. Generally speaking, the lmax in hexane, ethanol, and petroleum ether will show little if any change, but chloroform, for example, will show a shift to a longer wavelength. Other factors which may effect the spectral characteristics are water in water-miscible solvents, protein in carotenoproteins, and low temperatures.

Degradation of carotenoids

It must be remembered that carotenoids are sensitive to light, heat, air, and active surfaces; therefore, precautions must be taken during preparation and any subsequent storage to avoid any detrimental effects such as degradation, formation of stereoisomers, structural rearrangement, and other physicochemical reactions. The conditions of handling and any preparation prior to storage or extraction are critical to ensuring that there is no degradation of the analytes prior to analysis. Where possible, all manipulations during the preparation of standard solutions and extracts should be carried out under yellow/gold fluorescent lighting.

In certain instances, degradation can be rapid, thus it is essential that the chances of it occurring be lessened or avoided completely. This is particularly important in the case of standard carotenoid solutions. It has been reported that lycopene in particular can be rapidly degraded in chloroform from certain sources (Scott, 1992). With all standard solutions it is important not to assume that a standard has remained stable during storage.

Carotenoids in chlorophyll containing samples may be subject to chlorophyll sensitized photoisomerization, resulting in the production of significant amounts of *cis* (*Z)* isomers even during a brief exposure of an extract to light. As an example of a related problem, in acetone, *cis*-isomers can be produced as a result of the production of triplet state carotenes. The presence of O_2 in stored samples, peroxides in solvents, or oxidizing agents can rapidly lead to bleaching and carotenoid epoxides and apocarotenoids. Unsaturated lipids and metal ions can also enhance oxidative breakdown. Impurities such as plasticizers, especially phalates, can produce severe problems in spectrophotometric analysis, so all contact of samples, solvents and other reagents with plastic materials should be avoided wherever possible.

Carotenoids can be converted into mixtures of geometrical isomers under appropriate conditions, the most common being iodine catalyzed photoisomerization. This produces an equilibrium mixture of isomers, in general the *all-trans* isomers predominates. These isomers in an isomeric mixture cannot be measured separately by simple spectrophotometric determination. The usual method of subsequent measurement would be chromatographic separation, diode-array detection, and spectral analysis. In the absence of any definitive data on extinction coefficients for *cis*-isomers, they are quantified against the *all-trans* isomer. Modern procedures involve the direct synthesis of *cis*-carotenoids.

Extinction coefficients for carotenoid extracts

There are fewer problems if the extract contains essentially only one carotenoid, or if a single carotenoid has been collected from a chromatographic separation; however, most food extracts will contain at least two predominant carotenoids, and green vegetables in particular will also contain chlorophylls, which have absorption bands in the same region as the carotenoids. Plasma samples will contain a whole array of different carotenoids, although only five or six may predominate, but even these may, depending on the diet, be present at very different levels in different individuals, and some may even be absent; however, for plasmas, it may be a useful tool for screening individuals with "high" and "low" carotenoid

levels. Here again it does not give any information about individual carotenoids and samples indicating a "high" level may be biased in favor of one or two carotenoids.

The problem relating to chlorophylls can be overcome to some extent by saponification of the sample, which will remove the chlorophylls; however, care must be taken in the choice of conditions, as some carotenoids, particularly the xanthophylls, may be degraded (see *UNIT F2.1*). On the other hand, it is possible to use an alternate wavelength for the carotenoids. For example most of the major carotenoids of interest in foods have an absorption peak around 480 nm, where any absorption of chlorophylls causes less interference; however, it is then necessary to use alternate extinction coefficients (e.g., 2180 for β-carotene).

If the extract or fraction is composed of one predominant carotenoid, it can be monitored at the appropriate wavelength and the $A^{1\%}$ for that carotenoid used. If the extract is composed of more than one carotenoid, the absorbance at 450 nm can be measured and the A1% for β-carotene used. Results can be expressed as total β-carotene equivalents. Alternatively a "typical" $A^{1\%}$ value of 2500 can be used for comparing relative quantities between various sample extracts. An $A^{1\%}$ value of 2500 is appropriate since the predominate carotenoids, with the exception of lycopene ($A^{1\%}$ = 3450), have λ between 2460 and 2710. Of course if samples of tomatoes are to be analyzed, which contain lycopene predominantly, then an $A^{1\%}$ value of 3450 would be more appropriate.

It must be stressed that the value obtained in this way for mixtures of carotenoids is only an approximation. For more definitive analysis column chromatography (*UNIT F2.3*) should be used.

Anticipated Results

Using the extinction coefficients given above, the example for β-carotene, a 1% solution (1 g/100 ml or 10 mg/ml) would give a theoretical absorbance reading of 2592 AU. A 2 µg/ml solution should therefore give an absorbance reading of 0.518 AU; however, as indicated earlier, the standard solution must be analyzed by HPLC to determine the chromatographic purity (see Basic Protocol 1).

Time Considerations

The time taken to carry out the procedures outlined above will depend on the knowledge and experience of the analyst and their familiarity with the equipment; however, once familiar with the procedure, the preparation and calibration of a standard solution of a carotenoid (see Basic Protocol 1) should not take >1 hr.

The time required for Basic Protocol 2 will depend on the extraction procedure.

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Structural formula, common and chemical designations, and references.

Contributed by K. John Scott
Institute of Food Research
Colney, Norwich, United Kingdom

Chromatographic Techniques for Carotenoid Separation

This unit describes several liquid chromatographic techniques for separating and measuring carotenoids. The first protocol incorporates a reversed-phase separation using a wide-pore, polymerically-synthesized C18 column with visible detection at 450 nm (see Basic Protocol 1). The first alternate protocol is also isocratic C18 reversed-phase, but permits simultaneous analysis of retinol, tocopherols, and carotenoids using both a programmable UV-Vis detector and fluorescence detector, or a single diode-array detector (see Alternate Protocol 1). The second alternate protocol is oriented toward more detailed carotenoid analysis of geometric isomers (see Alternate Protocol 2); it incorporates a unique C30 "carotenoid" column with gradient separation and visible detection at 450 nm. The final basic protocol described in this unit is a normal-phase separation permitting more complete quantitation of xanthophylls and their isomers (see Basic Protocol 2). Two support protocols are described. The first details the preparation of standards for generating a calibration curve (see Support Protocol 1), while the second provides guidelines for sample preparation based on knowledge of the sample matrix (see Support Protocol 2).

ISOCRATIC CAROTENOID SEPARATION USING WIDE-PORE, POLYMERIC C18

BASIC PROTOCOL 1

This method requires the least sophisticated equipment and relies heavily on the unique characteristics of the column to separate the carotenoids (Craft et al., 1992; Epler et al., 1992). It incorporates the use of a polymeric C18 column, which has been shown to offer unique selectivity for structurally similar compounds such as geometric isomers. The addition of a second detector or use of a diode-array detector permits the simultaneous analysis of tocopherols, but not retinol. If the method is modified to incorporate a solvent gradient, retinol can be measured also (MacCrehan and Schonberger, 1987).

Materials

HPLC grade methanol
HPLC grade acetonitrile
HPLC grade triethylamine (TEA)
Calibration standards and (optional) internal standard (see Support Protocol 1)
Food sample of interest (see Support Protocol 2)

Vacuum filtration device, ultrasonicator, or inline vacuum degasser
HPLC system:
- Column: Vydac 201 TP or 218 TP C18 column, 5 µm, 250 × 4.6 mm (Vydac/Separations Group; preferred), Bakerbond WP C18 (J.T. Baker), or HiPore RP 318 (BioRad Laboratories) and guard column containing similar packing material
- Data recorder: computer data system or integrator
- Detector: fixed, variable, programmable, or diode-array (DAD) UV-Vis detector
- Injector: manual or automatic
- Pump: Isocratic

Prepare mobile phase

1. Prepare mobile phase by mixing 900 ml methanol, 100 ml acetonitrile, and 1 ml of triethylamine (TEA).

   Triethylamine serves as a modifier to prevent both nonspecific adsorption and oxidation.

Contributed by Neal E. Craft
Current Protocols in Food Analytical Chemistry (2001) F2.3.1-F2.3.15

2. Degas the mobile phase via vacuum filtration, ultrasonic agitation, or inline vacuum degasser.

Set HPLC conditions

3. Set the pump flow rate at 1.0 ml/min.
4. Set UV-Vis detector at 450 nm (436 nm if using filter photometer).
5. Inject individual standards and the standard mixtures, including any optional internal standard, as described to generate a standard curve (see Support Protocol 1).
6. Inject 10 to 50 µl of sample (see Support Protocol 2) and any optional internal standard dissolved in a solvent miscible with the mobile phase (e.g., ethanol, methanol, acetonitrile).

   The complete separation from lutein to lycopene requires ~20 min. Figure F2.3.1 illustrates the separation of carotenoid standards using this system. The elution order using this method (i.e., lutein, zeaxanthin, β-cryptoxanthin, echinenone, α-carotene, β-carotene, lycopene) differs from many other methods.

   Carotenoid retention and separation are influenced by column temperature; at temperatures above 20° to 25°C, lutein and zeaxanthin may not be well separated.

   Tocopherols can be measured simultaneously by using a diode array detector, a second UV detector set at 280 to 300 nm, or a fluorescence detector set at 296 nm excitation and 336 nm emission.

7. Calculate the final concentrations of carotenoids in samples by multiplying the peak areas of analytes by the calibration response factors. Apply sample weight and dilution factors to arrive at the concentration of carotenoids in the original sample (i.e., initial concentration).

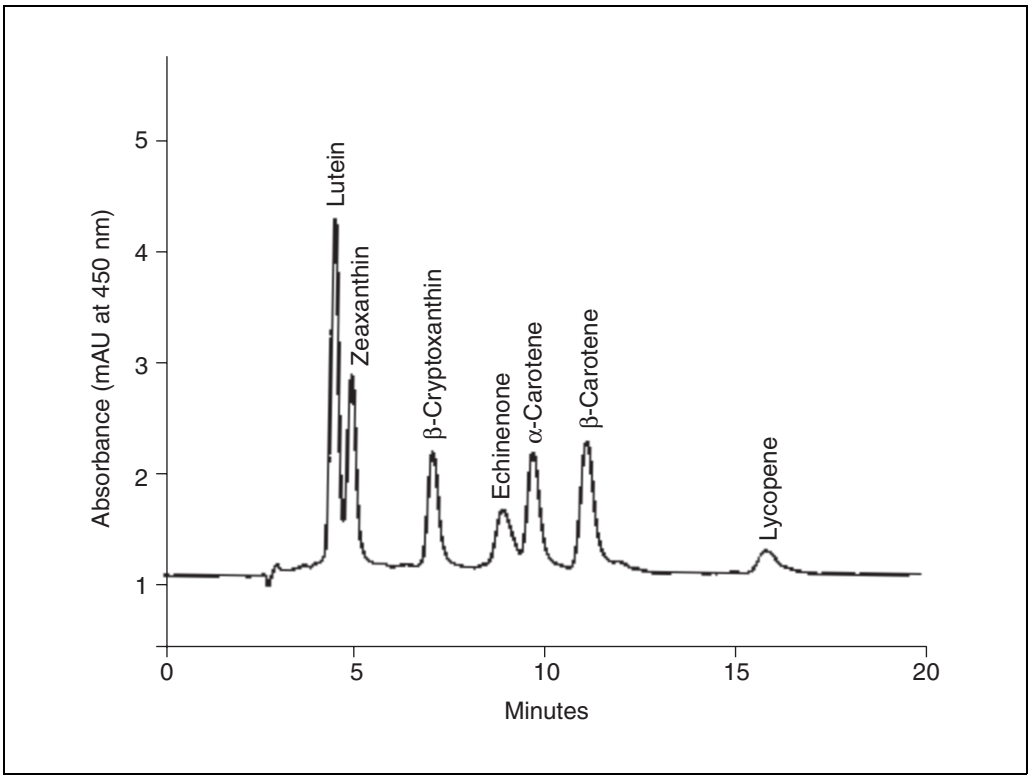

Figure F2.3.1 Isocratic HPLC separation of carotenoid standards using Basic Protocol 1. Conditions: 5-µm × 250-mm × 4.6-mm Vydac 201TP column, 90:10 methanol/acetonitrile mobile phase, 1.0 ml/min flow rate, visible detection at 450 nm, column temperature 25°C.

The response factor is the ratio of the analyte concentration to the peak area (or height) produced under the defined set of conditions during calibration (e.g., wavelength, solvent composition, column). Typically, data systems will generate response factors for each analyte from calibration data. For example, to calculate the initial concentration, where the sample weight (W1) is 0.5 g, the initial dilution (D1) is 25 ml, and the dilution factor for injection (D2) is 10×. The final concentration of the injected sample (Fc) equals the response factor multiplied by the peak area (or height). The initial concentration (Ic) equals the final concentration multiplied by the initial dilution multiplied by the dilution factor for injection divided by the sample weight (Ic = [Fc × D1 × D2]/W1 = [5.0 μg/ml × 25 ml × 10]/0.5 g = 2.5 mg/g, where Fc = Pa × Rf = 25,000 units × 0.002 = 5.0 μg/ml).

STANDARDS PREPARATION AND CALIBRATION

SUPPORT PROTOCOL 1

Analytical methods are only as good as the initial calibration; therefore, it is essential that the calibration for the accompanying HPLC methods be performed carefully. Carotenoids are labile compounds that are seldom obtained in pure form and degrade readily upon exposure to oxygen and light. Precautions should be taken to minimize standard and sample exposure to UV light and air. Given the above considerations, it is never recommended that carotenoid calibrants be prepared gravimetrically without verification using a spectrophotometer. This can lead to serious errors in quantitation (Craft et al., 1990). Many impurities contribute mass but not color to the standards. In addition, carotenoids tend to dissolve slowly in many solvents. Thus accuracy can be significantly improved by applying absorptivities ($E^{1\%}_{cm}$) and Beer's Law to filtered solutions of carotenoids to obtain concentration. The value assigned can be further refined by correcting for peak purity. This is accomplished by injecting individual standard solutions into the HPLC column while monitoring the wavelength maximum of each standard. Once the standard solution concentrations have been established, the individual carotenoid standard solutions can be mixed to form calibration solutions. The general procedure is provided below.

Materials

Crystalline carotenoid standards: lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene
Reagent alcohol with and without 30 ppm butylated hydroxytoluene (BHT)
HPLC grade tetrahydrofuran (THF) stabilized with 250 ppm BHT

Table F2.3.1 Commonly Used Absorptivity Values for Carotenoids and Their Corresponding Wavelength Maxima

Analyte	Wavelength (nm)	Absorptivity ($E^{1\%}_{cm}$)[a]
α-carotene	444	2800
β-carotene	452	2592
δ-carotene	456	3290
α-cryptoxanthin	445	2636
β-cryptoxanthin	452	2386
Lutein	445	2550
Lycopene	472	3450
Neoxanthin	439	2243
Phytoene	285	1250
Phytofluene	347	1577
Violaxanthin	443	2250
Zeaxanthin	452	2350

[a]Absorptivities listed above were taken from Bauernfeind (1981). For some carotenoids, $E^{1\%}_{cm}$ is provided in petroleum ether or hexane and is not significantly different from those in ethanol.

Internal standard(s)
HPLC grade ethanol or hexane

Vacuum filtration apparatus with 0.45-µm membrane
Spectrophotometer (285 to 456 nm)
Polypropylene or PTFE membrane, 0.45 µm for vacuum filtration

Preparation of calibration standards

1. Dissolve ~1 to 2 mg lutein, zeaxanthin, β-cryptoxanthin, and other xanthophylls directly in 100 ml reagent alcohol containing 30 ppm BHT. Dissolve ~1 to 2 mg lycopene, α-carotene, and β-carotene in 10 ml THF stabilized with BHT, then dilute to 100 ml with reagent alcohol.

   For normal-phase separations, solutions should be prepared using hexane.

   Crystalline carotenoid standards are available from Sigma, Indofine Chemical, Atomergic Chemetals, Fluka Chemical, Kemin Industries, and others.

2. Vacuum filter stock solutions through a 0.45-µm membrane to remove any undissolved material.

3. Measure the absorbance of the solutions at the wavelength maximum, as described in Table F2.3.1, against an appropriate blank (i.e., reagent alcohol) on a spectrophotometer. Dilute appropriately with reagent alcohol to measure between 0.5 and 1.0 AU.

4. Inject each standard individually into the appropriate HPLC column (see Basic Protocols 1 and 2 and Alternate Protocol 1 and 2), monitoring its wavelength maximum to determine the necessary purity correction.

   For example, if the area of the analyte peak constitutes 90% of the total peak areas, then the concentration calculated using spectrophotometric absorbance is adjusted to 90%.

5. (Optional) Inject the internal standard(s) to determine if there are any degradation products or impurities that may co-elute and absorb at the wavelength of the analytes, and to determine the purity correction of the internal standard.

   An internal standard is a compound that is not present in the sample, but is chemically and physically similar to the analytes of interest. A fixed quantity is incorporated into the calibration solutions. The same concentration of internal standard is added to the samples during extraction to compensate for analyte recovery and injection variability. As seen in Figure F2.3.1, Echinenone, which is not typically found in foods, is used as the internal standard. Unfortunately, compounds which may be used as internal standards for carotenoid analysis are not readily available commercially.

6. Mix standards in the range expected for each analyte in the sample matrix (see Table F2.3.2), optionally adding a fixed amount of internal standard. Dilute to a known volume (usually 5 ml) with ethanol to provide the desired final concentration.

Table F2.3.2 Calibration Range for Carotenoids

Analyte	Range (µg/ml)
α-carotene	0.05–5.0
β-carotene	0.05–10.0
β-cryptoxanthin	0.05–5.0
Lutein	0.05–10.0
Lycopene	0.05–10.0
Zeaxanthin	0.05–5.0

A minimum of 3 concentrations should be prepared although 5 concentrations weighted toward the lower end are preferred.

Total carotenoids in any single solution should not exceed 20 μg/ml.

For normal-phase separations use hexane rather than ethanol for dilution.

7. Inject mixture (volume as recommended in the protocols for the particular column and conditions; see Basic Protocols 1 and 2 and Alternate Protocols 1 and 2) and generate calibration curves.

Most HPLC instruments include computer data systems which automatically plot peak response versus concentration to generate response factors; however, if using an older system, the standard curve can be plotted manually. The calibration curves should be linear with a correlation coefficient of >0.98 and intersect very near the origin.

After calibration of the HPLC, a food-based quality control material or reference material should be analyzed on a routine basis to validate the performance of the method. The chromatograms illustrated in the following sections of this chapter are of a food matrix created from a mixture of baby foods and infant formula; however, a similar standard food reference material (SRM 2383) may be directly purchased from the National Institute of Standards and Technology (NIST).

SAMPLE PREPARATION

SUPPORT PROTOCOL 2

Food materials are variable and complex matrices. Knowledge of the sample matrix is critical for accurate carotenoid quantification. The type and chemical form of carotenoids and the composition of the food matrix are critical to the amount of sample preparation that is necessary prior to sample analysis. Many factors regarding the food matrix must be considered for efficient carotenoid extraction. For instance, the relative content of lipid to carotenoid in the food matrix influences the method of sample preparation. If both the lipid and carotenoid content are high (e.g., margarine), it may be possible to dilute the sample in an organic solvent that is miscible with the HPLC mobile phase for direct injection; however, when the lipid content of the sample is high and the carotenoid content is low, saponification is useful to separate the lipid (primarily triglycerides) from the carotenoids. Another factor is the form of the carotenoid that is present in the sample. Carotenes (hydrocarbon carotenoids) do not form ester linkages and can be directly extracted by homogenizing in the presence of lipophilic solvents (e.g., hexane, ethyl acetate, and toluene); however, the xanthophylls frequently form esters which will readily extract into lipophilic solvents. The xanthophylls are more easily quantified in the free form, which requires hydrolysis. Saponification (alkaline hydrolysis) is necessary to remove chlorophylls that are present in many foods because they can interfere with the detection of carotenoids. In other words, there is not one given sample preparation that will apply to all foods; therefore, to provide general guidelines, sample preparation has been broken into the following three categories. 1. Oil-based food samples containing only hydrocarbon carotenoids or nonesterified xanthophylls that are visibly yellow to red in color (e.g., margarine). 2. Direct extraction for food samples that do not contain xanthophyll esters or chlorophylls, and have a low lipid and high carotenoid content (e.g., carrots). 3. Saponification for food samples containing xanthophyll esters, chlorophylls, or high lipid and low carotenoid content (e.g., spinach, eggs).

Materials

Food sample
HPLC grade tetrahydrofuran (THF) containing 250 ppm BHT
Reagent alcohol
Magnesium carbonate
50:50 methanol/THF

10% (w/v) pyrogallol in reagent alcohol
40% (w/v) potassium hydroxide (KOH) in methanol
Nitrogen gas
Saturated NaCl (~6 M)
75:25 hexane/THF
Sodium sulfate

50-ml volumetric flask
0.45-µm filter (optional)
Tabletop centrifuge and appropriate swinging bucket rotor
Vacuum filtering apparatus and Whatman filter no. 42 paper
30- to 50-ml tube with cap
Shaking water bath or ultrasonic bath, 60°C
1.5 × 12–inch glass column with sintered glass frit or plugged with glass wool
Solvent evaporation apparatus (e.g., SpeedVac, rotary evaporator, nitrogen manifold, TurboVap)

For oil-based food samples containing only hydrocarbon carotenoids or nonesterified xanthophylls that are visibly yellow to red in color (e.g., margarine)

1a. Weigh 0.5 to 5.0 g of sample.

2a. Dissolve in 25 ml of THF stabilized with 250 ppm BHT, then dilute to volume in a 50-ml volumetric flask. Dilute the sample further with reagent alcohol until the carotenoid concentration is ~5 to 10 mg/liter.

The carotenoid concentration is approximated by diluting and measuring the absorbance at 450 nm on a spectrophotometer where 1 AU = ~4 mg/liter.

3a. (Optional) Filter through 0.45-µm filter prior to injection.

If the sample is free of particulate, it may be directly injected into the HPLC column for analysis.

For direct extraction of food samples that do not contain xanthophyll esters, chlorophylls, or have a low lipid and high carotenoid content (e.g., carrots)

1b. Homogenize ~5 g of ground sample with 10% (w/w) magnesium carbonate and 25 ml of 50:50 methanol/THF.

Lyophilized and dry samples should be reconstituted with water prior to extraction. They may require saponification if the samples contain chlorophylls or xanthophyll esters. Beadlet materials should be suspended in hot water, saponified, or enzymatically hydrolyzed before extraction. Follow the manufacturer's recommendations before analyzing by HPLC.

2b. Centrifuge in a swinging bucket rotor for 10 min at 6000 × *g* , 4 °C and remove the supernatant.

3b. Repeat steps 1b and 2b until the extracting solvent is colorless.

4b. Combine the extracts and vacuum filter through Whatman paper no. 42 to remove particles.

5b. Dilute to a known volume with reagent alcohol so that the THF represents <10% of the total solution (e.g., if total volume is 90 ml dilute to 500 ml).

6b. (Optional) Filter through 0.45-µm filter prior to injection.

If the sample is free of particulate, it may be directly injected into the HPLC column for analysis.

For saponification of food samples containing xanthophyll esters, chlorophylls, or high lipid and low carotenoid content (e.g., spinach, eggs)

1c. Proceed with a direct sample extraction as instructed in steps 1b to 5b.

2c. Transfer a 5-ml aliquot to a 30- to 50-ml capped tube for saponification.

3c. Add 1 ml of 10% (w/v) pyrogallol in reagent alcohol.

4c. Add 2 ml of 40% (w/v) KOH in methanol.

The final concentration of reagents in the mixed solution should be ~5% to 10% KOH and ≥ 1% pyrogallol.

5c. Flush the tubes with nitrogen gas and cap.

6c. Saponify the samples at 60°C for 1 hr in a shaking water bath or 30 min in an ultrasonic bath.

7c. After saponification, dilute the samples with 8 ml of saturated NaCl solution.

8c. Extract by vigorous mixing with 10 ml 75:25 hexane/THF.

Free xanthophylls, both endogenous and present in the saponified samples, are more polar and extract less efficiently into lipophilic solvents. Frequently, the addition of a polar organic solvent (tetrahydrofuran, methylene chloride, diethyl ether) is required to thoroughly extract them from the sample matrix and aqueous phase.

9c. Remove the upper phase.

10c. Repeat the extraction step until the upper phase is colorless.

For some samples the phases will not separate on standing and may require centrifugation to break emulsions.

11c. Wash the combined extract with water to remove traces of KOH and pyrogallol that may have been co-extracted.

12c. Place 3 in. solid sodium sulfate in the bottom of a 1.5 × 12–in. glass column plugged with sintered glass frit or plugged with glass wool. After removing the water, pass the extract through the sodium sulfate to remove any traces of water remaining.

13c. Remove the solvent from the extract using a solvent evaporation apparatus.

14c. Dissolve residue in a known volume of reagent alcohol to yield 5 to 20 mg/liter (an absorbance of 1.5 to 5.0 AU at 450 nm).

Samples extracted into strong organic solvents (hexane, ether, pet ether, ethyl acetate, etc.) must be transferred into a solvent miscible with the mobile phase. A small volume (e.g., 1 ml) of the organic extract should be evaporated under N_2 gas and dissolved in reagent alcohol. Further dilutions with alcohol may be necessary to obtain 5 to 10 mg/liter concentration before HPLC injection.

15c. (Optional) filter through 0.45-µm filter prior to injection.

If this sample is free of particulate, it may be directly injected into the HPLC column for analysis.

ALTERNATE PROTOCOL 1

ISOCRATIC CAROTENOID SEPARATION CAPABLE OF SIMULTANEOUS SEPARATION OF RETINOL AND TOCOPHEROL USING SPHERISORB ODS2

This method is the simplest approach for simultaneous carotenoid, retinol, and tocopherol analysis. Both the column and mobile phase have been chosen to provide efficiency and selectivity for the analysis of these components without the use of a gradient. The method uses the Spherisorb ODS2 column, in which the C18 chain is monomerically bound to the silica particles (i.e., the C18 chain binds at one site to the silica particles).

Additional Materials *(also see Basic Protocol 1)*

Ammonium acetate
p-dioxane
Triethylamine (TEA)
Calibration standards and (optional) internal standard (see Support Protocol 1)
Food sample of interest (see Support Protocol 2)

Vacuum apparatus and 0.45-µm polytetrafluoroethylene (PTFE) or polypropylene membrane
HPLC system (see Basic Protocol 1):
Column: 3-µm × 150-mm × 4.6-mm Spherisorb ODS2 (ES Industries, Phenomenex, or Waters) and guard column containing similar packing material

Prepare solutions

1. Dissolve 0.385 g ammonium acetate in 50 ml methanol.
2. Mix 830 ml acetonitrile, 130 ml *p*-dioxane, and 1 ml triethylamine (TEA).

   TEA serves as a modifier to prevent both nonspecific adsorption and oxidation.

3. While stirring, slowly add 40 ml methanol/ammonium acetate solution (step 1).
4. Vacuum filter through 0.45-µm PTFE or polypropylene membrane.

Set HPLC conditions

5. Set the pump flow rate at 1.5 ml/min.
6. Set UV-Vis detector at 450 nm (436 nm if using filter photometer).
7. Inject 10 to 30 µl individual standards and the standard mixtures, including any optional internal standard, as described to generate a standard curve (see Support Protocol 1).

   Column temperature should be maintained at a fixed temperature between 22° and 29°C in either a room with well regulated temperature, or a column oven for above ambient temperatures.

   If the injection solvent is of comparable solvent strength to that of the mobile phase, up to 30 ml may be used as the sample injection volume; however, if the injection solvent is more lipophilic than the mobile phase, the injection volume is limited to 10 ml.

8. Inject 10 to 30 µl standard or sample dissolved in a solvent miscible with the mobile phase (e.g., methanol, acetonitrile).

   The complete separation from retinol to β-carotene requires ~15 min. Figure F2.3.2 illustrates the separation of vitamins and carotenoids in the mixed food extract using this LC system. The elution order using this method is: lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene.

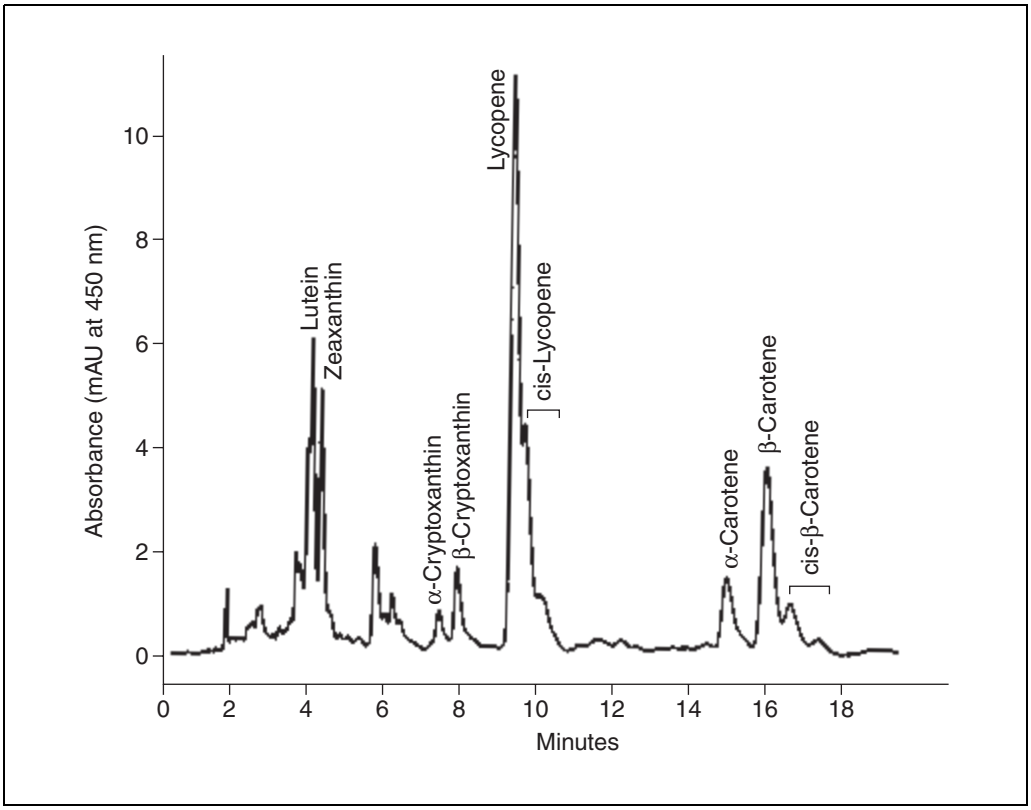

Figure F2.3.2 Isocratic HPLC separation of the food reference material carotenoids using Basic Protocol 2. Conditions: Spherisorb 3-μm × 150-mm × 4.6-mm ODS2 column, 83:13:4:0.1 acetonitrile/dioxane/150 mM ammonium acetate in methanol/TEA mobile phase, 1.5 ml/min flow rate, visible detection at 450 nm, column temperature 29°C.

Detection

9a. *For carotenoids:* Monitor 450 nm.

9b. *For retinol and carotenoids:* Monitor 325 nm for 3.2 min, then program the detector to change the wavelength to 450 nm.

10. *For tocopherols (optional):* Place fluorescence detector after UV-Vis detector and monitor excitation at 296 nm and emission at 336 nm.

11. *For diode array detectors (optional):* monitor 296 nm for tocopherols, 325 nm for retinol, and 450 nm for carotenoids.

12. Calculate the final concentrations as described above (see Basic Protocol 1, step 7).

GRADIENT SEPARATION USING C 30 CAROTENOID COLUMN

ALTERNATE PROTOCOL 2

This method requires the most sophisticated equipment and yields the most detailed results by utilizing the C30 column that was created specifically for carotenoid separation. The C30 column is polymerically bonded yielding selectivity similar to the polymeric C18. The column has an intermediate pore diameter (200 Å) and a 30 carbon alkyl chain, which results in higher carbon content and therefore stronger retention of the carotenoids. Due to the strong retention of carotenoids on this column, a gradient must be employed unless the sample is previously fractionated or only contains a specific group of carotenoids. It is possible to separate a wide polarity range of carotenoids and their isomers.

Additional Materials *(also see Basic Protocol 1)*

Calibration standards and (optional) internal standard (see Support Protocol 1)
Food sample of interest (see Support Protocol 2)
Mobile phase:
Solvent A: 0.05% TEA and 50 mM ammonium acetate in methanol
Solvent B: 0.05% TEA in isopropyl alcohol
Solvent C: 0.05% TEA in THF stabilized with 250 ppm BHT

HPLC system (see Basic Protocol 1)
Pump: ternary gradient
Column: 3-µm 250-mm × 4.6-mm C30 with 3-µ guard column (Waters)
Column oven: 35°C

Set HPLC conditions

1. Set the pump flow rate at 1.0 ml/min.
2. Set UV-Vis detector at 450 nm (436 nm if using filter photometer).
3. Inject 10 to 50 µl individual standards and the standard mixtures, including any optional internal standard as described to generate a standard curve (see Support Protocol 1).
4. Inject 10 to 50 µl sample (see Support Protocol 2) dissolved in a solvent miscible with the mobile phase (e.g., ethanol, 90% ethanol/10% isopropanol).

The complete separation from lutein to lycopene requires ~40 min with an additional 15 min to equilibrate the column back to the initial mobile phase. Figure F2.3.3 illustrates the separation of carotenoid in the mixed food extract using this LC system. The elution order

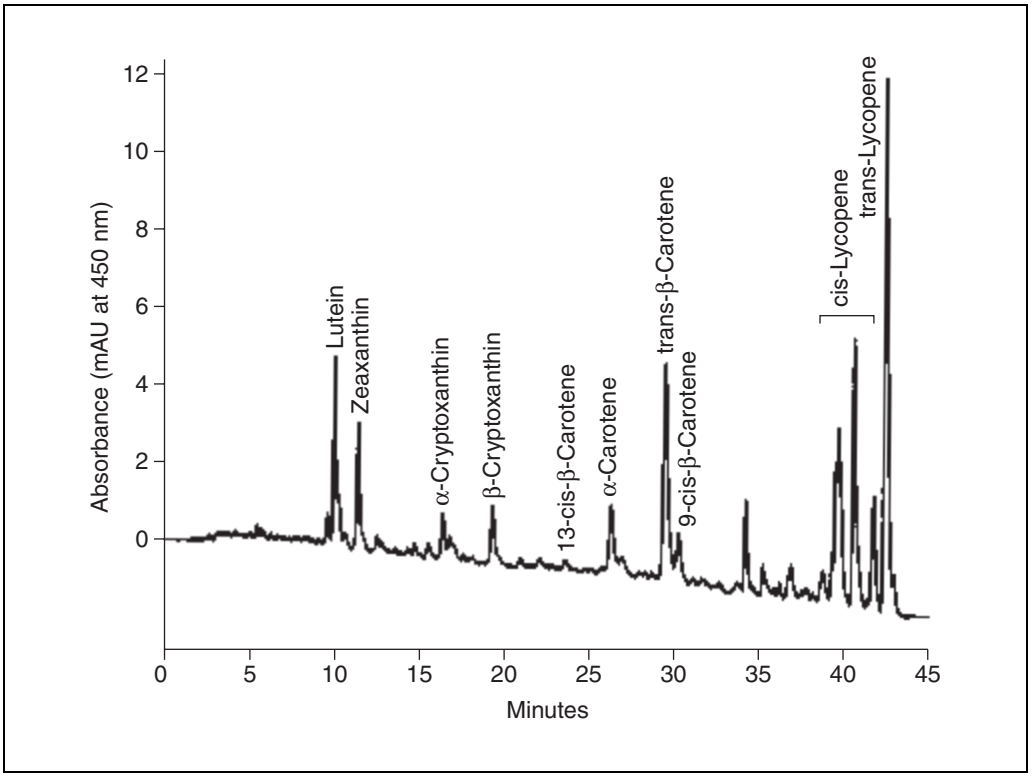

Figure F2.3.3 Gradient HPLC separation of the food reference material carotenoids using Protocol 3. Conditions: 3-µm × 250-mm × 4.6-mm Waters C30, column, 1.0 ml/min flow rate, visible detection at 450 nm, column temperature 35°C, solvent A = 50 mM ammonium acetate in methanol, B = isopropyl alcohol, C = tetrahydrofuran (all solvents contain 0.1% TEA). Flow program: 90% A/10% B linear gradient, 54% A/35% B/11% C over 24 min, linear gradient to 30% A/35% B/35% C over 11 min, hold 8 min, then return to initial conditions over 10 min.

using this method differs from many other methods: lutein, zeaxanthin, β-cryptoxanthin, 15-cis-β-carotene, 13-cis-β-carotene, α-carotene, trans β-carotene, 9-cis-β-carotene, cis-lycopene, and trans-lycopene.

Prepare gradient

5. Starting with 90% solvent A/10% solvent B, establish a linear gradient over 24 min to 54% solvent A/35% solvent B/11% solvent C, followed by a second linear gradient over 11 min to 30% solvent A/35% solvent B/35% solvent C. Hold 5 min, return to initial conditions over 10 min. Hold 5 min before next injection.

   Triethylamine in the solvent serves as a modifier to prevent both non-specific adsorption and oxidation.

Detect

6. *For carotenoids:* Monitor 450 nm.
7. *For tocopherols (optional):* Place fluorescence detector after UV-Vis detector and monitor excitation at 296 nm and emission at 336 nm.
8. *For a diode array detector (optional):* Monitor 296 nm for tocopherols, 325 nm for retinol, and 450 nm for carotenoids.
9. Calculate the final concentrations as described above (see Basic Protocol 1, step 7).

NORMAL-PHASE SEPARATION OF XANTHOPHYLLS

BASIC PROTOCOL 2

In normal-phase chromatography, polar components are more strongly retained than nonpolar components. Thus, hydrocarbon carotenes elute quickly while xanthophylls are retained and separated. This approach provides a more complete separation of polar carotenoids and their geometric isomers. This protocol is useful to the analyst that is specifically interested in the xanthophyll fraction of a sample.

Materials

Hexane
Dioxane
Indole-3-propionic acid (IPA)
TEA
Calibration standards and (optional) internal standard (see Support Protocol 1)
Food sample of interest (see Support Protocol 2)

Vacuum filtration device, ultrasonicator, or inline vacuum degasser
HPLC system (see Basic Protocol 1):
Column: 5-μm × 250-mm × 4.6-mm Lichrosorb Si column (ES Industries, Phenomenex, or EM Science) and guard column containing similar packing material

Prepare mobile phase

1. Prepare mobile phase by mixing 800 ml hexane, 200 ml dioxane, 15 ml IPA, and 2.0 ml TEA.

   TEA serves as a modifier to prevent both nonspecific adsorption and oxidation.

2. Degas the mobile phase via vacuum filtration, ultrasonic agitation, or inline vacuum degasser.

Set HPLC conditions

3. Set the pump flow rate at 1.0 ml/min.
4. Set UV-Vis detector at 450 nm (436 nm if using filter photometer).

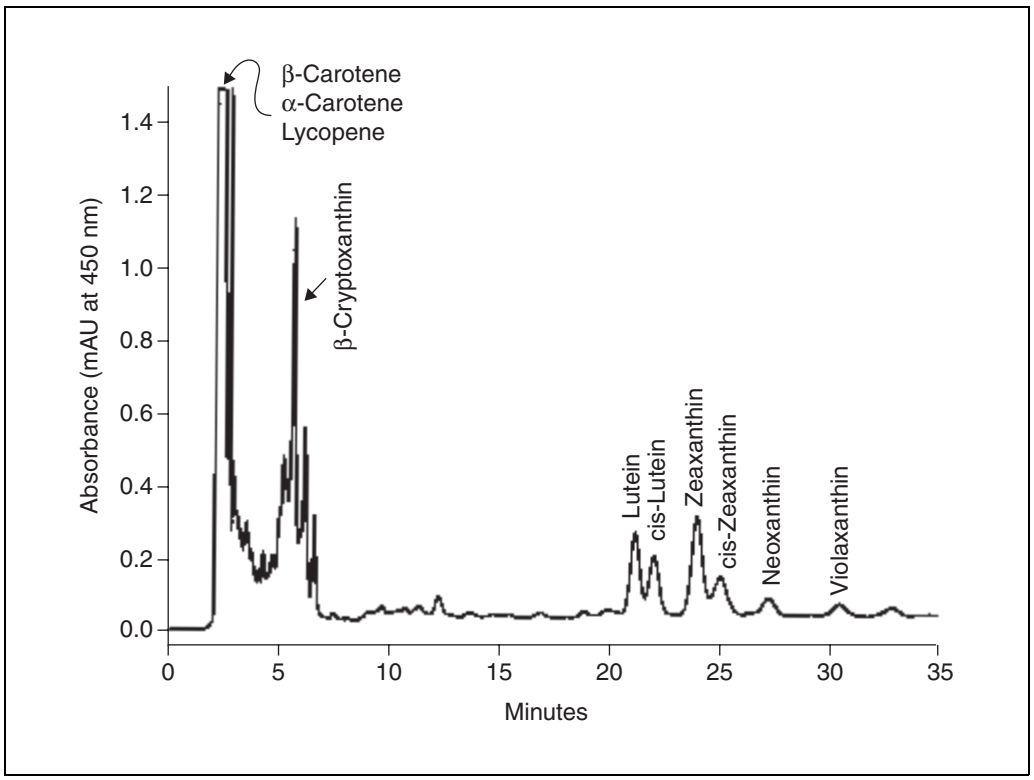

Figure F2.3.4 Isocratic HPLC separation of the food reference material carotenoids using Protocol 4. Conditions: Lichrosorb Si60, 5 μm, 250 × 4.6 mm column, hexane/dioxane/IPA/TEA (80:20:0.15:0.02) mobile phase, 1.0 ml/min flow rate, visible detection at 450 nm.

5. Inject 10 to 100 μl individual standards and the standard mixtures, including any optional internal standard as described to generate a standard curve (see Support Protocol 1).
6. Inject 10 to 100 μl of standard (see Support Protocol 1) or sample, including any optional internal standard, (see Support Protocol 2) dissolved in hexane.

   The complete separation from β-carotene to violaxanthin requires ~35 min. Figure F2.3.4 illustrates the separation of carotenoids in a mixed food extract using this LC system. The hydrocarbon carotenes (β-carotene, α-carotene, lycopene) elute together at the solvent front. The elution order is: β-cryptoxanthin, α-cryptoxanthin, lutein, cis-lutein, zeaxanthin, cis-zeaxanthin, neoxanthin, and violaxanthin.
7. Calculate the final concentrations as described above (see Basic Protocol 1, step 7).

COMMENTARY

Background Information

Carotenoids were among the first compounds to be separated by liquid chromatography, and actually inspired Mikael Tswett who coined the name meaning "color writing". Although the technique and instrumentation have evolved greatly since then, liquid chromatography is still the best mode of separating carotenoids. Until the 1970's all separations were performed using adsorption chromatography and low-pressure columns. The use of high-pressure pumps decreased analysis time and permitted the development of more efficient and novel column packing materials.

Carotenoid separations can be accomplished by both normal- and reversed-phase HPLC. Normal-phase HPLC (NPLC) utilizes columns with adsorptive phases (i.e., silica) and polar bonded phases (i.e., alkylamine) in combination with nonpolar mobile phases. In this situation, the polar sites of the carotenoid molecules compete with the modifiers present in the solvent for the polar sites on the stationary phase; therefore, the least polar compounds

elute first while the polar analytes, like the xanthophylls, are retained longer. Reversed-phase HPLC (RPLC), which is most commonly used for carotenoid analysis, incorporates nonpolar bonded phases, like the C18, or polymer phases, such as polystyrene-divinyl benzene, with polar mobile phases. The carotenoids partition between the nonpolar stationary phase and the polar mobile phase. Xanthophylls elute early due to their preference for the polar mobile phase while carotenes elute later due to their affinity for the stationary phase.

Several column factors are important for the separation of this very similar group of compounds. They include: the ligand, the ligand chain length, particle size, pore diameter, and method of ligand bonding. The ligand, which is bonded to the base silica, will determine the polarity of the column. The chain length is the number of carbon molecules in the ligand bonded to the silica and will influence analyte retention on the column. Particle size influences separation efficiency with smaller particles having a higher efficiency than larger particles. The pore diameter influences the carbon load of the column and thus, the retention of the analytes. The smaller the pore diameter, the greater the surface area and the higher the carbon load; however, if the pore diameter is too small, it will inhibit the large carotenoid molecules passage through the column. Finally, the method of ligand bonding refers to the synthesis used to bond the ligand to the base silica. The most common way of bonding the ligand is monomerically, in which the carbon chains are bonded to the silica surface using monochlorosilanes. The carbon chains extend into the pores of the silica and the analytes must pass by the individual chains on their way through the column. The second method is polymeric synthesis in which the carbon chains are bonded to the silica surface using trichlorosilanes in the presence of specific quantities of water. In this case, the carbon chains do not extend as individual finger-like projections into the path of the analytes, but the chains are cross-linked, forming a kind of irregular net that the analytes must pass through. Subtle changes from column to column in any of these factors will influence analyte separation. The reader should refer to the article by Craft (1992) for a detailed discussion of how these factors influence carotenoid separations.

The sensitivity of the method is influenced by the type of detector and lamp used in the detector. Deuterium lamps have weak energy in the wavelength range that carotenoids absorb, therefore the signal-to-noise is low. Tungsten and Xenon lamps have stronger energy in the 450-nm wavelength range and yield higher signal-to-noise ratios. Older diode-array detectors (DAD) tend to have poorer sensitivity than good programmable wavelength UV-Vis detectors due to the dispersion of light across the array of diodes. To improve sensitivity of a DAD, the bandwidth should be increased to 10 to 20 nm; however, to obtain good spectra of carotenoids, the bandwidth needs to be set at 1 to 5 nm, as the spectral difference between *cis* isomers is frequently only 2 to 4 nm. Newer DAD detectors have both deuterium and tungsten lamps providing better sensitivity for carotenoid analysis. In addition, at least one manufacturer incorporates a light pipe in the flow cell to improve sensitivity ~5-fold. Amperometric and coulometric detection of carotenoids have been used successfully (MacCrehan and Schonberger, 1987; Gamache et al., 1997). The advent of the coulometric array detector by ESA has permitted very low detection limits (picogram) along with a "finger print" profile. This mode of detection does require the use of a supporting electrolyte in the mobile phase (Ferruzzi et al., 1998). The addition of salts or ions to the mobile phase may alter elution profiles. Phytoene and phytofluene, which don't absorb at 450 nm, can be measured in each of the reversed-phase methods described by monitoring 285 nm and 345 nm, respectively. They elute with retention times in the area of hydrocarbon carotenoids.

Critical Parameters

Many parameters are critical to the successful and reproducible chromatographic separation of carotenoids. To assure stable baselines and consistent pump flow, solvents should be degassed before use to minimize outgassing during the gradient. Outgassing at the detector flow cell results in baseline noise. Solvents can be degassed using vacuum filtration through a 0.45-μ m filter, ultrasonic agitation for 15 min, helium sparging, or use of an inline solvent degasser. Use of a backpressure restrictor after the detector to maintain ~35 to 100 psi backpressure will also help to prevent this problem.

Due to the limited solubility and slow rate of dissolution of most carotenoids in organic solvents, be vigilant to avoid the use of solutions with high concentrations of carotenoids. The relative solubility of β-carotene and lutein in different solvents is discussed in Craft and Soares (1992). Calibration standards should be filtered through 0.45-μm membranes to re-

move any undissolved crystals. Tiny carotenoid crystals may precipitate out of stock solutions when placed at freezer temperatures. It is essential to dissolve the standard before use or filter the sample and reassign its concentration. Dissolution can be facilitated by agitation in an ultrasonic bath or warming the container in a 60°C water bath. When protected from light and oxygen, most stock and calibration solutions are stable at − 20°C for >6 months. Lycopene, however, degrades rapidly and is only stable ~1 week. Note that care should be taken to avoid directly exposing carotenoid stock solutions or samples to light, oxygen and acids since the carotenoids are labile to these conditions.

During the sample preparation, one must remember that the lipid behaves as a strong (lipophilic) solvent and in adequate concentration will cause peak broadening or doubling. In addition, dilution of these samples prior to injection must be great enough to prevent overloading the column with lipid. Thus, if the lipid content is high and the carotenoid content is low, the lipid (primarily triglycerides) must be separated from the carotenoid prior to HPLC analysis. Two general mechanisms can be used to remove the lipid: physical separation (solid phase extraction or liquid-liquid extraction) and hydrolysis (alkaline or enzymatic). Physical separation is only applicable under certain conditions since the solubility of hydrocarbon carotenoids is very similar to many lipids; however, if a sample contains high triglyceride and only polar carotenoids, the carotenoids could be partitioned into a polar organic solvent (e.g., methanol) or onto a polar solid phase extraction (SPE) cartridge (Si, NH_2 or CN) while the triglyceride remains with the nonpolar solvent (e.g., hexane). Saponification (alkaline hydrolysis), is the most common means of removing lipid from samples and has been described earlier (see Support Protocol 2). When saponification is used, it is essential to include antioxidants such as pyrogallol and ascorbic acid. Do not use BHT as it forms polymers when heated under alkaline conditions. The polymers absorb light in the visible range and co-elute with some carotenoids. Enzymatic hydrolysis requires the presence of a lipase to break down the triglyceride. Unfortunately, enzymes require an aqueous environment to function and the triglyceride is not soluble in water; therefore, the lipid forms a separate layer or oil droplets that provide limited surface area upon which the lipase can function.

Samples, even at moderate concentrations, injected into the HPLC column may precipitate in the mobile phase or at the column frit. In addition, the presence of other compounds (e.g., lipids) in the injection sample may drive the carotenoids out of solution or precipitate themselves in the mobile phase, trapping carotenoids. It is best to dissolve the sample in the mobile phase or a slightly weaker solvent to avoid these problems. Centrifugation or filtration of the samples prior to injection will prevent the introduction of particles that may block the frit, fouling the column and resulting in elevated column pressure. In addition to precipitation, other sources of "on-column" losses of carotenoids include nonspecific adsorption and oxidation. These can be minimized by incorporating modifiers into the mobile phase (Epler et al., 1993). Triethylamine or diisopropyl ethylamine at 0.1% (v/v) and ammonium acetate at 5 to 50 mM has been successful for this purpose. Since ammonium acetate is poorly soluble in acetonitrile, it should be dissolved in the alcoholic component of the mobile phase prior to mixing with other components. The ammonium acetate concentration in mobile phases composed primarily of acetonitrile must be mixed at lower concentration to avoid precipitation. In some cases, stainless steel frits have been reported to cause oxidative losses of carotenoids (Epler et al., 1992). When available, columns should be obtained with biocompatible frits such as titanium, Hastolloy C, or PEEK.

Not all C18 (ODS) columns are manufactured in the same way. As such, substitution of other columns for those listed in the above methods will probably yield inferior results. For a detailed explanation of column and solvent effects on the separation of carotenoids, see Epler et al. (1992).

Anticipated Results

During reversed-phase HPLC, carotenoids elute from the column in the order of polar to nonpolar. Reversed-phase chromatography tends to permit the elution of a wider range of components during a single isocratic separation. The three reversed-phase methods described herein should produce separations of the major carotenoids (i.e., lutein, α-cryptoxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene), in addition to components that are difficult to resolve, such as lutein/zeaxanthin and *cis*/*trans* isomers. Isocratic normal-phase chromatography is typically more appropriate for the separation of a narrow polarity

range of geometric and positional isomers. The elution order is from nonpolar to polar. A wider range of analytes can also be separated using a normal-phase gradient.

Time Considerations

Calibration is time consuming when performed correctly. It may require 1 or 2 days to perform all the necessary steps (i.e., prepare stocks, filter, measure absorbance, check purity, dilute, mix, and inject calibrants). Once the stock solutions and mixed calibration solutions have been prepared, a calibration check can be performed in ~4 hr. Sample preparation, depending on the matrix, may require a few minutes or a few hours. If an autosampler is unavailable for overnight injection the extracts are typically stable overnight, refrigerated at – 20° to 4°C. It is prudent to maintain the autosampler tray temperature from 4° to 15°C to reduce sample degradation. HPLC analysis of the extracted sample requires 20 to 60 min. Typically one technician can extract 12 to 24 samples per day to be analyzed overnight or the next day.

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Key References

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Mass Spectrometry of Carotenoids

The high sensitivity and selectivity of mass spectrometry (MS) facilitates the identification and structural analysis of small quantities of carotenoids that are typically obtained from biological samples such as plants, animals, or human serum and tissue. Structural information from the abundant fragmentation is provided by classical ionization methods, such as electron impact (EI; see Basic Protocol 1) and chemical ionization (CI; see Basic Protocol 1), but molecular ions are not always observed. Recent advances in soft ionization techniques, such as fast atom bombardment (FAB; see Basic Protocol 2), matrix-assisted laser desorption/ionization (MALDI; see Basic Protocol 3), electrospray ionization (ESI; see Basic Protocol 4), and atmospheric pressure chemical ionization (APCI; see Basic Protocol 5), have facilitated the molecular weight determination of carotenoids by minimizing fragmentation that is typical of EI and CI. Once the molecular weight of a carotenoid has been established using one of these ionization techniques, collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) can be used to augment fragmentation and obtain structurally significant fragment ions that may aid in the differentiation of structural isomers, such as differentiation of lutein from zeaxanthin, or of α-carotene from β-carotene and lycopene. CID and MS/MS parameters are independent of the ionization step so that no modifications of the sample preparation and ionization procedures are needed. Although CID and MS/MS can be used with any ionization technique, the application of this approach is illustrated using FAB ionization in Basic Protocol 2.

MS can be coupled to high-performance liquid chromatography (HPLC) to obtain separation of isomeric carotenoids or to remove interfering contaminants prior to ionization and detection. Except for MALDI, every ionization method discussed in this unit has been utilized during liquid chromatography MS (LC/MS; see Basic Protocols 1, 2, 4, and 5). However, LC-APCI-MS is now the preferred approach due to its widespread availability and ease of use. Although LC/MS and LC/MS/MS (see Basic Protocol 2) are routinely carried out, gas chromatography MS (GC/MS) is rarely used, because carotenoids typically decompose when exposed to the high temperatures of the GC process. Reviews of LC/MS and MS of carotenoids have been published by van Breemen (1996, 1997). A general introduction to mass spectrometry is given in Watson (1997).

ELECTRON IMPACT AND CHEMICAL IONIZATION MASS SPECTROMETRY OF CAROTENOIDS

BASIC PROTOCOL 1

Electron impact (EI) and chemical ionization (CI) MS have been used for carotenoid analysis for more than 30 years. Therefore, mass spectra of unknown carotenoids can be compared to a large number of published mass spectra to aid in identification. Unlike the newer "soft" ionization techniques in MS, EI and CI produce considerable fragmentation, and molecular ions are not always observed. Therefore, these techniques are most useful for obtaining fingerprints, or characteristic fragmentation patterns, instead of confirming or determining molecular weights of carotenoids. Alternatively, direct exposure EI (DEI) and/or direct exposure CI (DCI) MS can be used.

Materials

Carotenoid sample (1 to 100 mg/liter; 2 to 200 μM) dissolved in volatile organic solvent (e.g., hexane, tetrahydrofuran, methyl-*tert*-butyl ether, acetone), stored in an airtight glass vial

Reagent gas: methane or isobutane (for CI only)

Contributed by Richard B. van Breemen

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Microsyringe
Mass spectrometer equipped with direct insertion probe and electron impact (EI) and/or chemical impact (CI) ionization

NOTE: Carotenoid solutions degrade within hours at room temperature, but they can be stored(in many cases) ≤1 month at or below −20°C and 3 months at or below −70°C.

1. Using a microsyringe, load 1 µl carotenoid sample onto a direct insertion probe of a mass spectrometer.
2. Let solvent evaporate, then insert probe into the ion source of the mass spectrometer.
3. Introduce reagent gas (for CI only) and then turn on the electron beam (typically 70 eV for EI and 200 eV for CI).
4. Heat probe to vaporize carotenoid.
5. Record mass spectrum over the range *m/z* (mass-to-charge ratio) 50 to 800 for EI and *m/z* 100 to 800 for CI.

   All known carotenoids and their major fragment ions should be included in this range. By beginning the scan at m/z 100 during CI, reagent-gas ions can be avoided.

BASIC PROTOCOL 2

FAST ATOM BOMBARDMENT, LIQUID SECONDARY ION MASS SPECTROMETRY, AND CONTINUOUS-FLOW FAST ATOM BOMBARDMENT OF CAROTENOIDS

Fast atom bombardment MS (FAB-MS) and liquid secondary ion MS (LSIMS) are matrix-mediated desorption techniques that use energetic particle bombardment to simultaneously ionize samples such as carotenoids and transfer them to the gas phase for mass spectrometric analysis. Unlike with the EI and CI techniques, molecular ions are usually abundant and fragmentation is minimal.

Materials

3-Nitrobenzyl alcohol
Carotenoid sample (1 to 100 mg/liter; 2 to 200 µM) dissolved in volatile organic solvent (e.g., hexane, tetrahydrofuran, methyl-*tert*-butyl ether, acetone), stored in an airtight glass vial
HPLC solvents for LC/MS using continuous-flow FAB (e.g., methanol, methyl-*tert*-butyl ether)

Microsyringe
Mass spectrometer or tandem mass spectrometer equipped for fast atom bombardment (FAB)–MS or liquid secondary ion (LSI)MS, with direct insertion probe and with continuous-flow ionization source (as needed)
Syringe pump or HPLC pump capable of delivering flow rates of 1 to 10 µl/min (for continuous-flow only)
Reversed-phase HPLC (typically C18 or C30) column (for continuous-flow FAB-MS or LSIMS)

NOTE: The 3-nitrobenzyl alcohol matrix is typically stable at room temperature for ~3 months. Older solutions begin to turn yellow as they decompose and should be replaced. Carotenoid solutions will degrade within a few hours at room temperature but can be stored ≤1 month at or below −20°C and in some cases ≤3 months at or below −70°C.

NOTE: For LC/MS or flow injection using continuous-flow FAB, the mass spectrometer must be equipped with a continuous-flow ionization source.

NOTE: Details about chromatography columns used for carotenoids are contained in *UNIT F2.3*.

For probe analysis:

1a. Load 1 µl of 3-nitrobenzyl alcohol onto a direct insertion probe, then use a microsyringe to load 1 µl carotenoid sample onto the surface of the liquid matrix.

2a. Let solvent evaporate and then insert probe through the vacuum interlock into the ion source of a mass spectrometer or tandem mass spectrometer.

3a. Turn on FAB-MS or LSIMS beam and record the positive ion mass spectrum over the range *m/z* (mass-to-charge ratio) 50 to 900.

If desired, the peaks at m/z 154 and m/z 307 for the protonated monomer and dimer of the matrix, 3-nitrobenzyl alcohol, can be eliminated by scanning from m/z 310 to 900.

For continuous-flow analysis:

1b. Set up a syringe pump or an HPLC pump and equilibrate a reversed-phase HPLC column with appropriate eluents such as methanol and methyl-*tert*-butyl ether (e.g., 70:30, v/v).

A reversed-phase HPLC column (typically C18 or C30) is required for HPLC separations. Because the flow rate into the continuous-flow FAB-MS or LSIMS source must be <10 µl/min, either a capillary column must be used or else the flow must be split postcolumn. For narrow-bore HPLC columns operated at 200 µl/min, the split ratio would be 30:1. Isocratic or gradient separations may be used. A syringe pump is usually necessary for capillary columns, but standard HPLC pumps are sufficient for applications using narrow-bore columns.

2b. Add the matrix, ~0.1% (v/v) 3-nitrobenzyl alcohol prepared in appropriate mobile phase, postcolumn at a flow rate of ~1 to 3 µl/min.

3b. Interface the continuous-flow probe to a mass spectrometer. Tune the continuous-flow FAB-MS or LSIMS ion source on the 3-nitrobenzyl alcohol dimer ion at *m/z* 307.

4b. Inject a carotenoid sample onto the HPLC column, turn on the FAB-MS or LSIMS beam, and record the positive ion mass spectrum over the range *m/z* 300 to 1000.

An injection volume of 1 to 5 µl is typical for narrow-bore columns.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY OF CAROTENOIDS

BASIC PROTOCOL 3

A matrix-mediated ionization technique, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS uses an intense flash of laser light to vaporize a solid matrix containing the sample. Although usually regarded as an ionization method reserved for high-mass compounds such as proteins and polymers, MALDI has shown remarkable promise for the analysis of carotenoids. In particular, MALDI has been effective in the ionization of intact esterified carotenoids that would fragment too extensively using other ionization methods.

Materials

Carotenoid sample (1 to 100 mg/liter; 2 to 200 µM) dissolved in acetone, store in airtight glass vial

Acetone saturated with 2,5-dihydroxybenzoic acid (sample matrix)

Microsyringe

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer with UV laser (i.e., 337-nm nitrogen laser), MALDI probe, and optional delayed extraction and postsource decay

NOTE: Carotenoid solutions degrade rapidly at room temperature (within several hours) but can be stored for at least 1 month at or below −20°C and ~3 months at or below −70°C in some cases.

1. Mix 20 µl carotenoid sample with 10 µl acetone saturated with 2,5-dihydroxybenzoic acid.
2. Using a microsyringe, load 5 to 10 µl carotenoid/matrix sample onto the target of a MALDI probe.
3. Let solvent evaporate (only a few seconds are required) and then insert probe through the vacuum interlock into the ion source of a MALDI-TOF mass spectrometer.
4. Record MALDI-TOF mass spectra in positive ion mode. Look for molecules ions and protonated molecules in the range *m/z* 300 to 1000.

BASIC PROTOCOL 4

ELECTROSPRAY IONIZATION LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY OF CAROTENOIDS

Unlike other LC/MS techniques in which removal of the mobile phase and sample ionization are discrete steps, electrospray is both an ionization method and an interface between an HPLC system and a mass spectrometer. Electrospray is also one of the most universal ionization techniques for MS, as virtually every class of compound has been analyzed using this technique, including carotenoids.

For flow injection analysis of carotenoids, ~1 µl of a 0.1 to 100 µM carotenoid sample can be injected into a solvent stream and carried into the electrospray source without chromatography. This approach is particularly useful for high-throughput analysis of pure samples and for tuning and optimizing the parameters of the electrospray source. The sample should be dissolved in a volatile organic solvent (e.g., hexane, tetrahydrofuran, methyl-*tert*-butyl ether, acetone, methanol), but may contain water. The sample should be stored in an airtight glass vial. A narrow scan range (e.g., *m/z* 520 to 620 for compounds such as β-carotene and lutein) or selected ion monitoring of the molecular ions (e.g., *m/z* 536 and 568 for β-carotene and lutein, respectively) is usually used during flow-injection analysis.

When HPLC is used as part of the analysis, the mobile phase is typically a mixture of methanol and methyl-*tert*-butyl ether (i.e., 50:50, v/v), although other HPLC solvents for LC/MS using electrospray (e.g., water, tetrahydrofuran) can be used. It is important to note that entirely organic solvent systems might pose a fire hazard for some home-built ion sources and some older commercial instruments if air leaks into the ionization chamber. Therefore, water or a halogenated solvent should be added to the mobile phase postcolumn to suppress ignition. The electrospray source must always be vented outside the laboratory. The scan range is typically *m/z* 300 to 1000 in order to include known carotenoids and this esters.

When tuning the electrospray source of a mass spectrometer or tandem mass spectrometer, optimum sensitivity for carotenoids will be obtained using the highest possible voltage on the electrospray needle before corona discharge occurs. For example, an electrospray needle voltage of −5100 V provided excellent sensitivity in one published report (van Breemen, 1995). Because not all electrospray ion sources support voltages this high, the sensitivity might be lower in other systems. A reversed-phase HPLC C18 or C30 column is typically used for LC/MS with electrospray ionization. The flow rate into the mass spectrometer, as controlled by a syringe pump or HPLC pump, should be matched to the design of the system, but in most current commercial systems the flow rate can be varied

between 1 and 1000 µl/min. Microbore, narrow-bore, or analytical-scale columns at flow rates from 1 to 1000 µl/min are compatible with most LC/MS electrospray interfaces. Details about chromatography columns used for carotenoids are contained in *UNIT F2.3*.

2,2,3,4,4,4-Heptafluoro-1-butanol can be added postcolumn to give a final concentration of 0.1% (v/v) to enhance ionization efficiency during electrospray. Typically, a 2% (v/v) solution in mobile phase is added at 50 µl/min to the HPLC column effluent at 1 ml/min. Addition of this reagent is optional.

ATMOSPHERIC PRESSURE CHEMICAL IONIZATION LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY OF CAROTENOIDS

BASIC PROTOCOL 5

Most mass spectrometers equipped for electrospray ionization can be converted to APCI, and many commercial LC-APCI-MS instruments are equipped with both ionization techniques. During APCI, ionization takes place in an atmospheric pressure chamber when the sample molecules collide with solvent ions formed in a continuous corona discharge. Unlike electrospray, the needle used to spray the HPLC effluent is not at high voltage.

For flow-injection analysis of carotenoids, ~1 to 5 µl of a 0.1 to 100 µM carotenoid sample, which may contain water, can be injected into a solvent stream and carried into the APCI source without chromatography. This approach is particularly useful for high-throughput analysis of pure samples and for tuning and optimizing the parameters of the APCI source. The sample should be dissolved in a volatile organic solvent (e.g., hexane, tetrahydrofuran, methyl-*tert*-butyl ether, acetone, methanol), but may contain water. The sample should be stored in an airtight glass vial. A narrow scan range of *m/z* 520 to 620 is appropriate for most carotenoids such as β-carotene and lutein. Alternatively, selected in monitoring of the molecular ions or protonated or deprotonated molucules may be used.

When HPLC is used as part of the analysis, the mobile phase is typically a mixture of methanol and methyl-*tert*-butyl ether (i.e., 50:50, v/v), although other HPLC solvents for LC/MS using APCI (e.g., water, tetrahydrofuran) can be used. It is important to note that if combustible nonaqueous solvent systems are used, water or a halogenated solvent such as methylene chloride or chloroform should be added to the mobile phase postcolumn to suppress ignition in the ion source. In addition, the APCI source must be vented outside the laboratory and should not allow air into the ionization chamber. A scan range of *m/z* 300 to 1000 will include the known carotenoids and their most common esters.

A reversed-phase HPLC C18 or C30 narrow-bore column is typically used for LC/MS with APCI. Details about chromatography columns used for carotenoids are contained in *UNIT F2.3*. For most APCI systems, the optimum flow rate into a mass spectrometer or tandem mass spectrometer equipped with APCI, as controlled by a syringe pump or HPLC pump, is usually between 100 and 300 µl/min, which is ideal for narrow-bore HPLC columns. Larger diameter columns should be used with a flow splitter postcolumn to reduce the solvent flow into the mass spectrometer. For example, if a 4.6 mm i.d. column was used at a flow rate of 1.0 ml/min, then the flow must be split postcolumn ~5:1 so that only 200 µl/min enters the mass spectrometer.

COMMENTARY

Background Information

EI and CI

Most of the original structural elucidation studies of the >600 known carotenoids used EI and CI (Moss and Weedon, 1976). Although these ionization methods are usually used with GC during GC/MS, carotenoids are too thermally labile to pass through the hot oven of a GC and must be introduced into the MS using a direct insertion probe. Most EI and CI mass spectra of carotenoids have been acquired using positive ion mode, but negative ions may be formed during CI with electron capture. For example, McClure and Liebler (1995a,b) used negative ion chemical ionization to analyze oxidation products of β-carotene. EI and CI are gas-phase ionization techniques, which means that carotenoids must be volatilized prior to ionization using thermal heating. This process results in some pyrolysis prior to ionization and promotes fragmentation after ionization, which explains why molecular ions are not always observed in EI and CI mass spectra.

EI and CI have been used in combination with LC/MS in a technique called particle-beam LC/MS. During particle-beam LC/MS, the LC eluate is sprayed into a heated, near-atmospheric pressure chamber to evaporate the mobile phase, and the resulting sample aerosol is separated from the lower-molecular-weight solvent molecules in a momentum separator. Next, the sample aerosol enters the MS ion source, where the aggregates strike a heated metal surface and disintegrate into gas-phase sample molecules that are ionized by EI or CI. To minimize fragmentation and enhance sensitivity during particle-beam LC/MS, negative ion electron capture CI is usually used for carotenoid ionization. For example, Khachik et al. (1992) used particle-beam LC/MS with negative ion electron capture CI to analyze polar carotenoids extracted from human serum. They observed abundant molecular anions, $M^{-\bullet}$, and simple fragmentation patterns, such as loss of water from the molecular ion for xanthophylls like lutein.

FAB-MS and LSIMS

In widespread use since 1982 (Barber et al., 1982), FAB-MS and LSIMS are matrix-mediated techniques. Although matrices such as glycerol or thioglycerol are suitable for hydrophilic compounds such as peptides, a more hydrophobic matrix is required for the analysis of the nonpolar carotenoids. The most effective matrix for carotenoid ionization is 3-nitrobenzyl alcohol (Caccamese and Garozzo, 1990; Schmitz et al., 1992). Ionization and desorption of the carotenoid analyte occur together during the bombardment of the matrix by fast atoms (or ions) to produce primarily molecular ions, $M^{+\bullet}$. Unlike most FAB-MS and LSIMS analyses, protonated or deprotonated molecules of carotenoids are not usually observed. In addition, fragmentation is minimal compared to EI, CI, or MALDI.

Because molecular ions dominate FAB-MS and LSIMS mass spectra of carotenoids, collision-induced dissociation (CID) can be used in a tandem mass spectrometer to enhance the formation of structurally significant fragment ions. These ions provide information about carotenoid functional groups, such as the presence of hydroxyl groups, esters, or rings, or the extent of conjugation of the polyene chain. For example, the most abundant fragment ion in the tandem mass spectrum of β-carotene corresponds to loss of a neutral molecule of toluene, $[M\text{-}92]^{+\bullet}$, and indicates the presence of extensive conjugation within the molecule. This ion is also abundant in the tandem mass spectra of α-carotene, γ-carotene, lycopene, astaxanthin, neurosporene, β-cryptoxanthin, zeaxanthin, and lutein. Because the toluene molecule originates within the conjugated polyene chain and not from a terminus of these molecules, compounds such as neoxanthin, phytoene, or phytofluene, which have disruptions in their polyene chains, do not fragment to eliminate toluene. Instead, phytoene and phytofluene exhibit a fragment ion of $[M\text{-}94]^{+\bullet}$.

For example, the ion of $[M\text{-}69]^{+\bullet}$, which is observed in the tandem mass spectra of lycopene, neurosporene, and γ-carotene but not α-carotene, β-carotene, lutein, or zeaxanthin, indicates the presence of a terminal acyclic isoprene unit. Elimination of a hydroxyl group or a molecule of water, $[M\text{-}17]^{+\bullet}$ or $[MH\text{-}18]^{+}$, from carotenoids such as astaxanthin or zeaxanthin is characteristic of the presence of a hydroxyl group. Also, tandem mass spectrometry can be used to distinguish between isomeric carotenoids such as α-carotene and β-carotene, or lutein and zeaxanthin. For example, the ring of α-carotene containing the double bond that is not conjugated to the rest of the polyene chain shows unique retro-Diels-Alder fragmentation to form the ion of $[M\text{-}56]^{+\bullet}$. In a similar manner, isomeric lutein and zeaxanthin differ by the

position of the same carbon-carbon double bond that distinguishes α-carotene and β-carotene. Consequently, only lutein fragments to eliminate this unusual ring and form an ion of *m/z* (mass-to-charge ratio) 428.

The fragmentation patterns and characteristic fragment ions for the carotenoids observed in FAB-MS and LSIMS tandem mass spectra are also observed in the tandem mass spectra obtained following ESI (see Basic Protocol 4), APCI (see Basic Protocol 5), and other methods. A detailed account of structure determination of carotenoids using FAB ionization with CID and MS/MS is presented in van Breemen et al. (1995). Finally, another advantage of MS/MS is that matrix ions formed during FAB-MS or LSIMS, and any other contaminating ions, are eliminated, which simplifies interpretation of the mass spectrum.

FAB ionization has been used in combination with LC/MS in a technique called continuous-flow FAB LC/MS (Schmitz et al., 1992; van Breemen et al., 1993). Although any standard HPLC solvent can be used, including methyl-*tert*-butyl ether and methanol, the mobile phase should not contain nonvolatile additives such as phosphate or Tris buffers. Volatile buffers such as ammonium acetate are compatible at low concentrations (i.e., ≤10 mM). Continuous-flow FAB has also been used in combination with MS/MS (van Breemen et al., 1993). The main limitations of continuous-flow FAB compared to other LC/MS techniques for carotenoids, such as ESI and APCI, are the low flow rates and the high maintenance requirements. During use, the 3-nitrobenzyl alcohol matrix polymerizes on the continuous-flow probe tip causing loss of sample signal. As a result, the continuous-flow probe must be removed and cleaned approximately every 3 hr.

MALDI-TOF

MALDI-TOF-MS facilitates the analysis of carotenoids and other natural products with detection limits that are lower than most other techniques. For example, subpicomole quantities can be detected (Wingerath et al., 1999). The enhanced sensitivity is the result of the efficiency of the pulsed ionization and detection system in which a complete mass spectrum is recorded with each laser flash. Like FAB and LSIMS, molecular ions are the most abundant sample ions, although some protonated molecules and $[M\text{-}H]^+$ ions may be formed as well. Abundant molecular ions of carotenoid esters have been observed using MALDI-TOF-MS (Kaufmann et al., 1996; Wingerath et al., 1996), which is significant because most other desorption ionization techniques, including FAB, ESI, and APCI, tend to produce molecular ions of esters in lower abundance with abundant deesterified fragment ions. MALDI-TOF-MS is not yet compatible with on-line chromatographic systems, so techniques such as continuous-flow FAB, ESI, or APCI should be selected when LC/MS is required.

ESI

During electrospray ionization, the HPLC eluate is sprayed through a capillary electrode at high potential (usually 2000 to 7000 V) to form a fine mist of charged droplets at atmospheric pressure. As the charged droplets are electrostatically attracted towards the opening of the MS, they encounter a cross-flow of heated nitrogen that increases solvent evaporation and prevents most of the solvent molecules from entering the MS. Although ions produced by electrospray ionization are usually preformed in solution by acid-base reactions (i.e., $[M+nH]^{n+}$ or $[M-nH]^{n-}$), carotenoid ions are probably formed by a field desorption mechanism at the surface of the droplet, which appears to be enhanced by the presence of halogenated solvents such as heptafluorobutanol (van Breemen, 1995). As a result of this unusual ionization process, electrospray ionization of carotenoids produces abundant molecular cations ($M^{+\bullet}$) with little fragmentation, and no molecular anions.

Because of the efficiency of the solvent removal in the LC/MS interface and the flexibility of the ESI process, ESI is compatible with a wide range of HPLC flow rates (from 0.1 nl/min to 1 ml/min) and with a variety of mobile phases including the methanol/methyl-*tert*-butyl ether solvent system that is ideal for separations using C30 carotenoid columns. A potential limitation of ESI for quantitation is its relatively narrow dynamic range (approximately two orders of magnitude). Aside from this narrow range of linear response during quantitative analysis, ESI shows excellent sensitivity and is compatible with HPLC using a wide range of solvents and flow rates. An example of the positive ion LC-ESI-MS analysis of carotenes in an extract of human plasma is shown in Figure F2.4.1.

APCI

APCI uses a heated nebulizer to facilitate solvent evaporation and obtain a fine spray of the mobile phase instead of a strong electromagnetic field as in electrospray. Unlike elec-

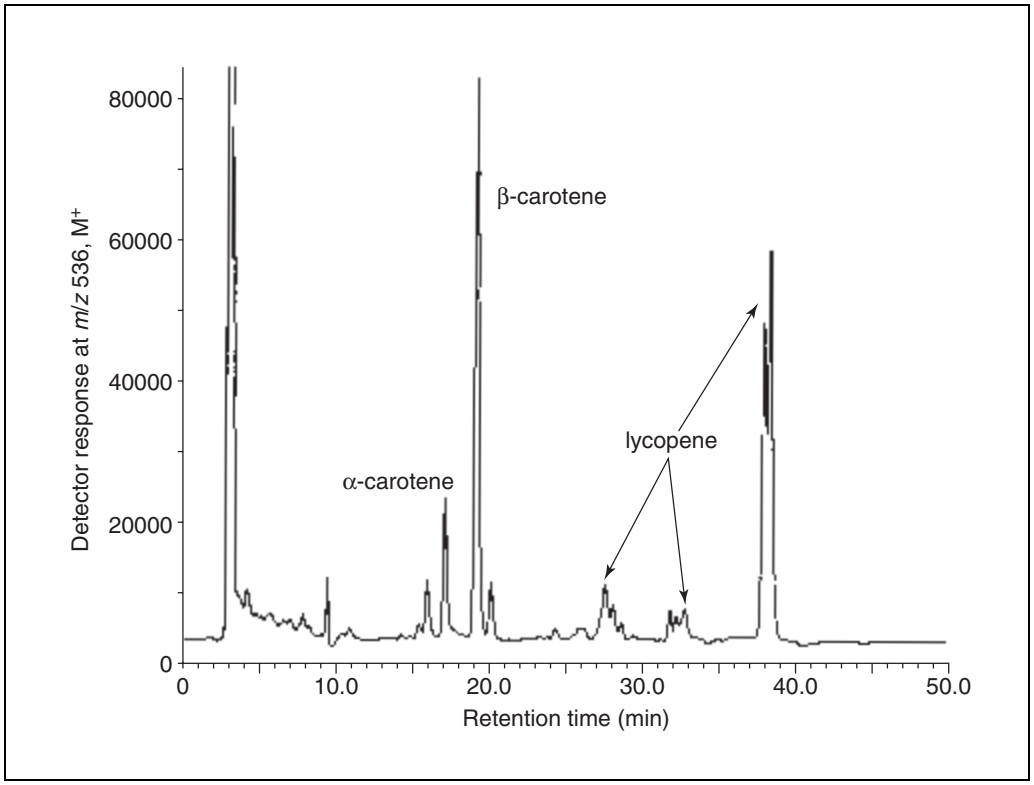

Figure F2.4.1 Liquid chromatography/mass spectrometry (LC/MS) analysis of isomeric carotenes in a hexane extract from 0.5 ml human serum. Positive ion electrospray ionization MS was used on a quadrupole mass spectrometer with selected ion monitoring to record the molecular ions of lycopene, β-carotene, and α-carotene at *m/z* (mass-to-charge ratio) 536. A C30 HPLC column was used for separation with a gradient from methanol to methyl-*tert*-butyl ether. The all-*trans* isomer of lycopene was detected at a retention time of 38.1 min and various *cis* isomers of lycopene eluted between 27 and 39 min. The all-*trans* isomers of α-carotene and β-carotene were detected at 17.3 and 19.3 min, respectively.

trospray, the nebulizer is not surrounded by a strong electromagnetic field. Ionization takes place by spraying the mobile phase containing analyte into a corona discharge in an atmospheric pressure chamber. The corona discharge ionizes the solvent gas (a chemical reagent gas) that ionizes the carotenoid analyte through ion-molecule reactions. Ions are then drawn into the aperture of the MS for analysis.

The main advantage of APCI compared to ESI for carotenoid analysis is the higher linearity of detector response (exceeding four orders of magnitude of carotenoid concentration), which suggests that LC-APCI-MS should be the preferred MS technique for carotenoid quantitation. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations, and abundant fragmentation, which tends to reduce the abundance of the molecular ions. Like ESI, APCI provides high sensitivity for carotenoids with detection limits near 1 pmol injected on-column during LC/MS. The superior linearity of the MS response during APCI of carotenoids suggests that this LC/MS technique may become the standard for carotenoid quantitation.

Critical Parameters

During EI and CI of carotenoids, the ion source of the MS should be maintained at a low temperature because carotenoids are heat labile. A typical low source temperature would be 100°C. Higher source temperatures can be used, but will result in fewer molecular ions and increased fragmentation.

The choice of matrix for FAB-MS, LSIMS, and MALDI is essential for efficient sample ionization. For example, the use of 3-nitrobenzyl alcohol instead of glycerol, thioglycerol, or most other more common matrices is essential for the formation of abundant carotenoid ions during FAB and LSIMS. Nonpolar carotenoids (e.g., the carotenes) are insoluble in polar ma-

trices such as glycerol or thioglycerol and ionize inefficiently. During MALDI, the matrix and carotenoid sample must be dissolved in the same solvent. Usually, acetone is suitable for this purpose. Furthermore, the matrix must absorb strongly at the wavelength of the incident laser beam. Although a UV laser and UV-absorbing matrix are described, other types of lasers and matrices can be used.

The mobile phase for ESI and APCI should be volatile and can include all common HPLC solvents and compositions. Nonvolatile mobile-phase additives such as nonvolatile buffers or ion-pair agents should be avoided, because these compounds will precipitate and contaminate the ion source. During electrospray, volatile buffers such as ammonium acetate, ammonium formate, and ammonium carbonate can be used at concentrations <40 mM. At higher concentrations, even volatile buffer ions will suppress electrospray ionization. In contrast, volatile buffers at concentrations exceeding ~40 mM will not suppress ionization during APCI, although they may clog the nebulizer or block the entrance to the MS if their evaporation is too slow relative to their concentration.

Troubleshooting

Because extensive fragmentation is typical of EI and CI mass spectra, molecular ions or protonated molecules might not be observed. In order to confirm the molecular weight of a carotenoid, desorption EI or desorption CI (also known as in-beam EI and CI) can be utilized to increase the abundance of the molecular ion species. If the molecular weight of the carotenoid remains uncertain, then softer ionization techniques should be investigated, such as FAB-MS, ESI, MALDI, or APCI.

The limits of detection for carotenoids using FAB-MS and LSIMS are not as low as with most other ionization techniques (Schmitz et al., 1992). Therefore, ≥10 pmol of each carotenoid should be loaded onto the direct insertion probe per analysis. The matrix, 3-nitrobenzyl alcohol, has been effective in facilitating the ionization of all types of carotenoids. However, more polar matrices such as glycerol or thioglycerol might be useful for the FAB-MS or LSIMS analysis of polar xanthophylls such as astaxanthin. Because glycerol and thioglycerol are poor solvents for hydrophobic compounds, they are unlikely to solvate and thus facilitate the ionization of the nonpolar carotenes such as β-carotene.

Whether using FAB-MS, LSIMS, or MALDI, matrix ions might interfere with the detection of certain carotenoids. Therefore, alternate but chemically similar matrices with different molecular weights might need to be utilized on a case-by-case basis.

Anticipated Results

EI and CI

Abundant fragment ions will be observed below *m/z* 300 due to random cleavage of carbon-carbon bonds. Therefore, these ions are of little value in structure elucidation. Fragment ions above *m/z* 300 will be useful in confirming the presence of specific ring systems and functional groups. Internal cleavage to eliminate toluene, $[M-92]^{+\bullet}$, is an abundant ion for most carotenoids including β-carotene, α-carotene, and lycopene. For example, β-carotene and zeaxanthin eliminate toluene to form fragment ions of *m/z* 444 and *m/z* 476, respectively. This fragmentation pathway indicates the presence of an extensive conjugated carbon-carbon double bond system. For comparison, the incompletely conjugated carotene phytoene does not form an abundant fragment ion corresponding to $[M-92]^{+\bullet}$. As discussed above (see Troubleshooting), molecular ions or protonated molecules will usually be observed in low abundance.

FAB-MS and LSIMS

Molecular ions, $M^{+\bullet}$, will be observed with almost no fragmentation. For example, the positive ion FAB-MS mass spectrum of the carotene phytofluene is shown in Figure F2.4.2. The base peak of *m/z* 542 corresponds to the molecular ion. For the more polar xanthophylls, protonated molecules may be observed as well as molecular ions. During bombardment, sample ions will be continuously produced for several minutes until the sample or the matrix is consumed. During this time, exact mass measurements and high resolution measurements can be carried out to determine carotenoid elemental compositions. If structurally significant fragment ions are desired, then CID can be used to fragment the molecular ions followed by MS/MS to record the resulting product ions.

MALDI

Abundant molecular ions, $M^{+\bullet}$, will be observed as the base peaks in the MALDI-TOF mass spectra. Delayed extraction will enhance the abundance of the molecular ions relative to background noise, and the use of postsource decay will facilitate the detection of structurally

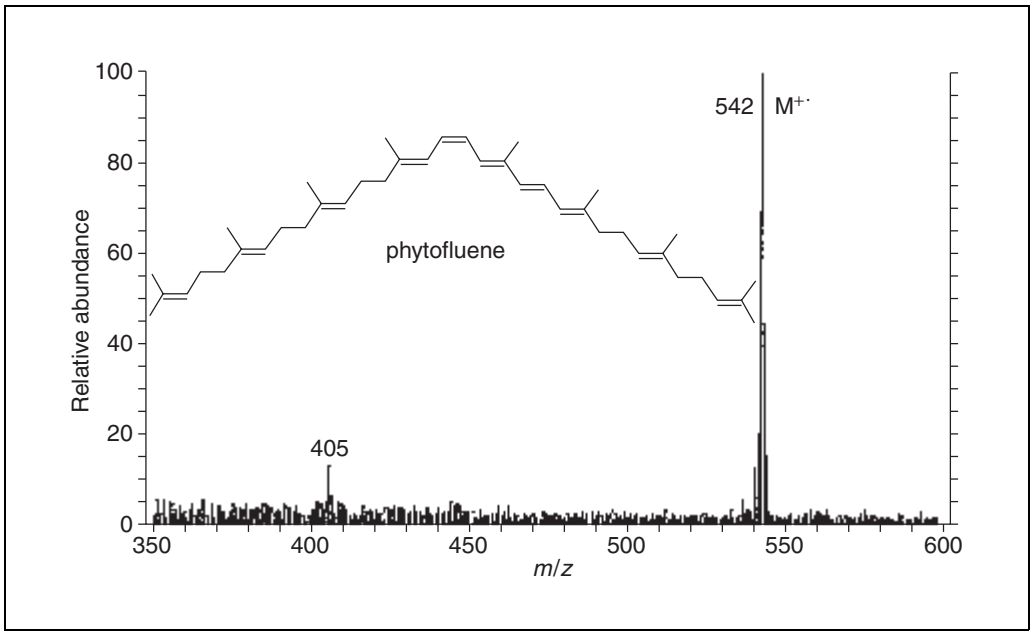

Figure F2.4.2 Positive ion fast atom bombardment (FAB-MS) mass spectrum of phytofluene isolated from blueberries. The base peak of *m/z* (mass-to-charge ratio) 542 corresponds to the molecular ion. Characteristic of FAB-MS, background signals are observed at every *m/z* value. The mass spectrum was obtained during continuous-flow FAB-MS LC/MS using a magnetic sector mass spectrometer. Although the 16-*cis* isomer of phytofluene is shown, the FAB mass spectra of the all-*trans* and other *cis* isomers are indistinguishable.

significant fragment ions that resemble the product ions observed during MS/MS with CID (Kaufmann et al., 1996; Wingerath et al., 1996). Delayed extraction and postsource decay are available on many commercial MALDI-TOF mass spectrometers. Delayed extraction is simply the introduction of a variable pause, usually milliseconds in length, between the firing of the laser during ionization and the translocation of the sample ions from the source to the time-of-flight mass analyzer. This delay allows high-energy and low-mass matrix ions and fragment ions to disperse and be quenched, leaving primarily the sample molecular ions in the ion source for subsequent analysis. Postsource decay is the process of analyzing metastable ions (or fragment ions formed outside the ion source) using a reflectron type of time-of-flight mass spectrometer. This type of analysis is similar to MS/MS, and the types of carotenoid fragment ions that are observed are analogous to those obtained using MS/MS with CID.

ESI

Electrospray ionization will produce molecular ions, $M^{+\bullet}$, with almost no fragmentation for carotenes and many xanthophylls. As the polarity of the carotenoid increases, the probability of forming protonated (positive ion mode) or deprotonated (negative ion mode) molecules will increase. Negative molecular ions of carotenoids, $M^{-\bullet}$, have not been reported using electrospray. The LC/MS analysis of isomeric carotene weighing 536 Da is shown in Figure F2.4.1. For this analysis, hydrophobic carotenoids including lycopene, α-carotene, and β-carotene were extracted from human serum using hexane and then separated on a narrow-bore C30 reversed-phase HPLC column using a mobile-phase gradient from methanol to methyl-*tert*-butyl ether. Molecular ions were formed using ESI and the signals for all ions of *m/z* 536 are shown in the computer-reconstructed mass chromatogram in Figure F2.4.1. The all-*trans* isomers of α-carotene, β-carotene, and lycopene were detected at retention times of 17.3 min, 19.3 min, and 38.1 min, respectively. The other peaks of *m/z* 536 eluting between 27 and 39 min corresponded to *cis* isomers of lycopene. In addition, *cis* isomers of α-carotene and β-carotene were observed eluting near the all-*trans* isomers between 15 and 21 min.

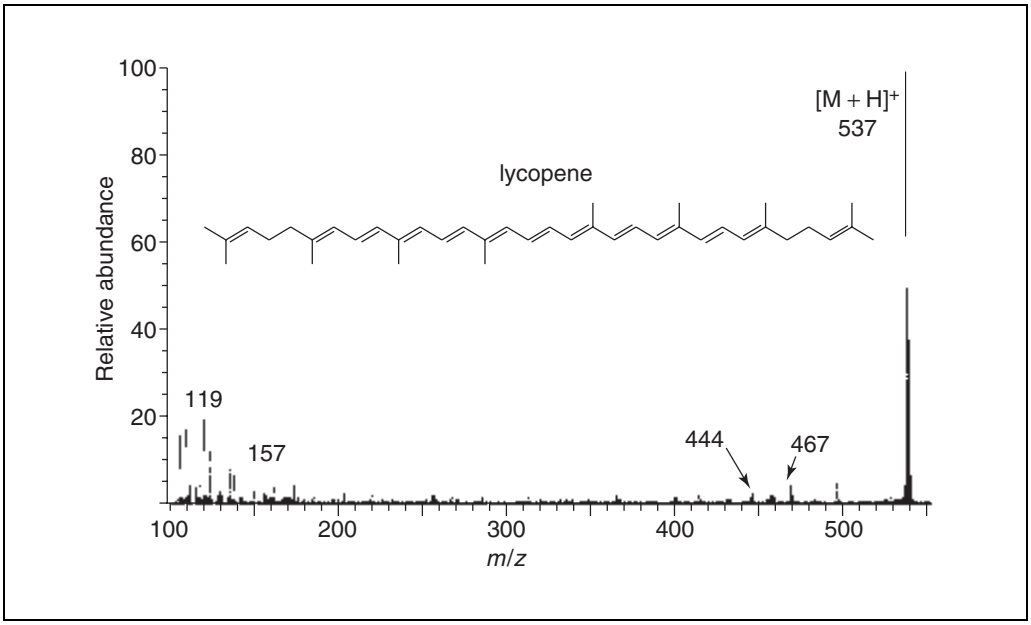

Figure F2.4.3 Flow-injection positive ion atmospheric pressure chemical ionization (APCI) mass spectrum of ~1 pmol lycopene. The carrier solvent for flow injection analysis consisted of methanol/methyl-*tert*-butyl ether (50:50; v/v) at a flow rate of 200 µl/min. The lycopene standard was isolated from tomatoes. The all-*trans* isomer of lycopene is shown, which is the most abundant isomer found in the tomato. This carotene is the familiar red pigment of the tomato.

APCI

Carotenoids form both molecular ions and protonated molecules during positive ion APCI, and molecular ions and deprotonated molecules during negative ion analysis. The relative abundances of molecular ions and protonated or deprotonated molecules vary with the mobile-phase composition (van Breemen et al., 1996). For example, polar solvents such as alcohols lead to an increased abundance of protonated carotenoids, and nonpolar solvents such as methyl-*tert*-butyl ether facilitate the formation of molecular ions. Even though APCI tends to produce more fragmentation in the ion source than either ESI or FAB-MS, these fragment ions are often not abundant. For example, the positive ion APCI mass spectrum of lycopene is shown in Figure F2.4.3. If additional fragmentation is desired for structure confirmation, then CID with MS/MS would be required.

The elemental composition and exact mass of many common carotenoids is shown in Table F2.4.1.

Time Considerations

Because MS is a rapid analytical technique, the sample throughput can exceed 120 samples per hour. Specifically, the throughput of EI, CI, and FAB-MS is typically up to 20 samples per hour, and the rate-limiting step is usually the minute or two required to introduce the direct insertion probe or sample target containing each sample through the vacuum interlock. Although up to 60 analyses per hour may be achieved using MALDI, these rapid measurements are only possible after an array of samples has been loaded into the ion source of the mass spectrometer. Most commercial MALDI-TOF mass spectrometers allow multiple samples to be loaded onto a single sample stage, thereby increasing the efficiency of sample introduction into the mass spectrometer. Therefore, the rate-limiting step for MALDI-TOF-MS is sample preparation, which includes mixing samples with matrix solutions, depositing each solution on the MALDI target, allowing the solvent to evaporate, and then introducing the target through a vacuum interlock into the ion source of the mass spectrometer.

During chromatographic analysis, such as particle-beam LC/MS, continuous-flow FAB-MS or LSIMS, and LC/MS using ESI or APCI, the rate-limiting step is the chromatographic separation. Most analytical-scale HPLC separations require 15 to 30 min each, which limits the throughput to only two or four samples per hour. However, the high throughput demanded

Table F2.4.1 Elemental Compositions and Exact Masses[a] of Common Carotenoids[b]

Carotenoid	Elemental composition	Exact mass
β-Apo-8′-carotenal	$C_{30}H_{40}O$	416.3079
3-Hydroxy-β-apo-8′-carotenal	$C_{30}H_{40}O_2$	432.3028
α-Carotene	$C_{40}H_{56}$	536.4382
β-Carotene	$C_{40}H_{56}$	536.4382
γ-Carotene	$C_{40}H_{56}$	536.4382
Lycopene	$C_{40}H_{56}$	536.4382
Neurosporene	$C_{40}H_{58}$	538.4539
ς-Carotene	$C_{40}H_{60}$	540.4695
Phytofluene	$C_{40}H_{62}$	542.4852
Phytoene	$C_{40}H_{64}$	544.5008
2′,3′-Anhydrolutein	$C_{40}H_{54}O$	550.4175
Echinenone	$C_{40}H_{54}O$	550.4175
α-Cyrptoxanthin	$C_{40}H_{56}O$	552.4331
β-Cryptoxanthin	$C_{40}H_{56}O$	552.4331
Alloxanthin	$C_{40}H_{52}O_2$	564.3967
Canthaxanthin	$C_{40}H_{52}O_2$	564.3967
Diatoxanthin	$C_{40}H_{54}O_2$	566.4124
Lutein	$C_{40}H_{56}O_2$	568.4280
Isozeaxanthin	$C_{40}H_{56}O_2$	568.4280
Zeaxanthin	$C_{40}H_{56}O_2$	568.4280
Lycopene-16,16′-diol	$C_{40}H_{56}O_2$	568.4280
4-Ketoalloxanthin	$C_{40}H_{50}O_4$	578.3760
Pectenolone	$C_{40}H_{52}O_3$	580.3916
Phoenicoxanthin	$C_{40}H_{52}O_3$	580.3916
4-Ketozeaxanthin	$C_{40}H_{54}O_3$	582.4073
Antheraxanthin	$C_{40}H_{56}O_3$	584.4229
Lutein epoxide	$C_{40}H_{56}O_3$	584.4229
7,8,7′,8′-Tetradehydroastaxanthin	$C_{40}H_{48}O_4$	592.3553
7,8-Didehydroastaxanthin	$C_{40}H_{50}O_4$	594.3709
Astaxanthin	$C_{40}H_{52}O_4$	596.3866
Neoxanthin	$C_{40}H_{56}O_4$	600.4179
Isozeaxanthin bispelargonate	$C_{58}H_{88}O_4$	848.6683

[a]Exact mass is defined as the monoisotopic molecular weight of a molecule and is calculated using the mass of the most abundant isotope of each element.
[b]Moss and Weedon (1976) contains a more extensive table of carotenoids and their molecular weights.

by combinatorial chemistry has resulted in the development of HPLC methods utilizing shorter HPLC columns and higher mobile phase flow rates, such that the throughput can exceed 60 LC/MS analyses per hour using ESI or APCI. When chromatographic separations are not required, flow injection ESI- or APCI-MS analyses can be carried out with throughputs exceeding 10 samples per minute or >600 samples per hour.

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Key References

Moss and Weedon, 1976. See above.

Extensive molecular ion and fragment ion abundances are listed for positive ion EI mass spectra of carotenoids.

van Breemen, R.B. 1995. Electrospray liquid chromatography-mass spectrometry of carotenoids. *Anal. Chem.* 67:2004-2009.

An important early reference describing ionization conditions for carotenoid analysis using electrospray MS.

van Breemen et al., 1993. See above.

Describes the selection and optimization of matrix and mobile-phase conditions for carotenoid analysis using LC-MS/MS with FAB-MS ionization.

van Breemen et al., 1995. See above.

Contains the most complete set of tandem mass spectra of carotenoids and their interpretation. Fragmentation patterns and characteristic fragment ions described here are common to all CID tandem mass spectra of carotenoids.

van Breemen et al., 1996. See above.

The original reference for LC-APCI-MS of carotenoids, containing essential information for carrying out C30 HPLC separations with on-line APCI mass spectrometric detection.

Wingerath et al., 1999. See above.

Describes sample preparation and matrix requirements for analyzing carotenoids using MALDI-TOF MS and discusses the use of postsource decay for obtaining structurally significant fragment ions of carotenoids during MALDI-TOF MS.

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Betalains

Betalains, the pigments of the red beet, have found increased use as food colorants. This unit describes a multiple-component system using visible spectrophotometry to determine the concentration of the pigment components (i.e., betacyanins and betaxanthins) calculated in terms of betanin and vulgaxanthin-I, respectively (see Basic Protocol 1). The total pigment content (betalain) is expressed as the sum of betacyanins and betaxanthins.

In the first protocol (see Basic Protocol 1), the maximum light absorption of betanin, the major betacyanin, and vulgaxanthin-I, the major betaxanthin, are measured. The betanin and vulgaxanthin contents are calculated using each pigment's 1% absorptivity value $A^{1\%}$. The method takes into account small amounts of interfering substances.

A second protocol is presented in which the betacyanin pigments are first separated into their individual components and then quantified by applying each pigment's $A^{1\%}$ (see Basic Protocol 2). This technique is particularly useful when measuring pigment content in heat-treated, partially-degraded, or stored colorant mixtures, as large amounts of degradation products will interfere with the spectrophotometric method.

In addition, a protocol describing the extraction of betalains from beets is provided (see Support Protocol).

SPECTROPHOTOMETRIC DETERMINATION OF BETACYANINS AND BETAXANTHINS

BASIC PROTOCOL 1

This protocol describes a spectrophotometric method for measuring the betacyanin and betaxanthin content in beet juice or beet tissue extract (see Support Protocol). Light absorption measured at 538 nm and 476 nm is used to calculate the betanin and vulgaxanthin-I concentrations, respectively. In addition, the absorption at 600 nm is measured and used to correct for small amounts of impurities. Since no prior separation of the pigments is made, the light absorption measurement at A_{538} and A_{476} includes all minor betacyanins and betaxanthins, respectively. The results are expressed as betacyanin (calculated in terms of betanin) and betaxanthin (calculated in terms of vulgaxanthin-I). The total betalain concentration is expressed as the sum of the betacyanins and betaxanthins.

Materials

0.05 M phosphate buffer, pH 6.5: 4/9.4 (v/v) 8.863 g/liter Na_2HPO_4/6.773 g/liter KH_2PO
Beet juice or tissue extract (see Support Protocol)
Spectrophotometer and appropriate recording device
1-cm path-length quartz cuvette

1. Turn on the spectrophotometer and allow the instrument to warm up for at least 30 min before taking measurements.
2. Zero the spectrophotometer at 476, 538, and 600 nm using 0.05 M phosphate buffer, pH 6.5 as the solvent blank.
3. Dilute beet juice or tissue extract with 0.05 M phosphate buffer, pH 6.5 such that the A_{538} of the sample is between 0.4 and 0.5 AU.

See Critical Parameters for a discussion about the narrow range of absorbance units.

Contributed by Joachim H. von Elbe
Current Protocols in Food Analytical Chemistry (2001) F3.1.1-F3.1.7

4. Obtain the visible absorption spectrum of the solution between 450 and 650 nm in recorded form and read the absorption directly at 538, 476, and 600 nm.

5. Calculate the corrected light absorption of betanin and vulgaxanthin-I using the follow set of equations:

$$x = 1.095 \times (a - c)$$
$$z = a - x$$
$$y = b - z - x/3.1$$

Where

a = light absorption of the sample at 538 nm

b = light absorption of the sample at 476 nm

c = light absorption of the sample at 600 nm

x = light absorption of betanin minus the colored impurities

y = light absorption of vulgaxanthin-I corrected for the contribution of betanin and colored impurities

z = light absorption of the impurities.

The derivation of these working equations is discussed elsewhere (see Critical Parameters).

6. Calculate the betanin and vulgaxanthin-I concentrations using each pigment's $A^{1\%}$ and applying the appropriate dilution factor (step 3).

The absorptivity value ($A^{1\%}$) is the extinction coefficient representing a 1% solution (1.0 g/100 ml) and is 1120 for betanin and 750 for vulgaxanthin-I (Wyler and Dreiding, 1957; Piattelli and Minale, 1964).

As an example, if a 1-ml sample of centrifuged beet juice was diluted to 150 ml with buffer, and the absorption spectrum obtained resulted in a light absorption at 538, 476, and 600 nm of 0.422 (a), 0.378 (b), and 0.052 AU (c), respectively, then applying the above equations results in x (betanin) = 0.405, y (vulgaxanthin-I) = 0.230, and z (impurities) = 0.017. Using $A^{1\%}$ for each pigment and the dilution factor of 150×, betanin and vulgaxanthin-I concentrations in the juice of 54 mg/100 ml and 46 mg/100 ml are derived, respectively.

BASIC PROTOCOL 2

QUANTIFICATION OF INDIVIDUAL BETACYANINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

This protocol describes the separation of individual betacyanin pigments (i.e., betanin, isobetanin, betanidin, and isobetanidin) by HPLC, and the quantification of each by comparing the peak area of each pigment to the peak area of a standard curve or collecting each fraction of known volume and applying the respective $A^{1\%}$. The pigments are separated using either isocratic or gradient separation conditions.

Materials

Solvent A: 18/82(v/v) CH_3OH/0.05 M KH_2PO_4: adjust to pH 2.75 with H_3PO_4
Solvent B: CH_3OH
Sephadex G-25 (Amersham Pharmacia Biotech) water slurry, pH 2.0: adjust pH with HCl
Sample of beet juice or beet tissue extract (see Support Protocol), pH 2.0: adjust pH with HCl
1% acetic acid
Four 7.88-mm × 61-cm Bondapak C_{18}/Porasil B columns connected in series
17.8/81.2/1.0 (v/v/v) CH_3OH/0.05 M KH_2PO_4/acetic acid
0.1% HCl

Standard analytical or preparatory HPLC:
Variable wavelength detector
Injection loop: 10 ml (preparatory) or 20 µl (analytical)
Column: reversed-phase Bondapak C_{18}/Porasil B (Water Associates)
1000-ml gel filtration column (Amersham Pharmacia Biotech)
Freeze drier
0.45-µm HA filter (Millipore)
Table top centrifuge 25°C

Initialize HPLC gradient

1. Set the flow rate of the HPLC at 8 ml/min at room temperature. If using a gradient, set the initial mixture to 100% solvent A, changing over 9 min to 80% solvent A, 20% solvent B.

   If an isocratic separation is desired, use 100% solvent A.

Prepare standards

2. Slurry pack a 1000-ml gel filtration column with a Sephadex G-25 water mixture, pH 2.0.
3. Apply 150 ml beet juice or tissue extract, pH 2.0.
4. Elute the column with 500 ml 1% acetic acid.
5. Collect red (betacyanin) fraction and freeze dry.
6. Dissolve freeze dried material in a minimum of double-distilled water, filter through a 0.45-µm HA-Millipore filter and chromatograph by preparatory LC—i.e., place ten

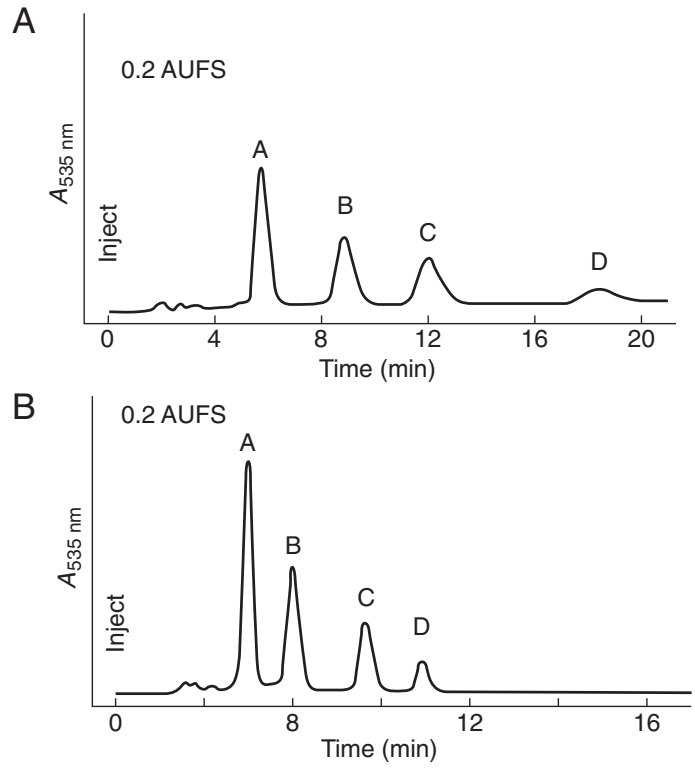

Figure F3.1.1 Isocratic gradient LC chromatogram of betacyanin pigments. Peak A: betanin; peak B: isobetanin; peak C: betanidin, and peak D: isobetanidin. Adopted from Schwartz and von Elbe (1980).

samples on four 7.88-mm × 61-cm Bondapak C_{18}/Porasil B columns connected in series. Elute columns with 17.8/81.2/1.0 (v/v/v) CH_3OH/KH_2PO_4/acetic acid using a flow rate of 8 ml/min.

7. Collect both the betanin and betanidin fraction (~50 ml each; Fig. F3.1.1). Place each fraction into a centrifuge tube and maintain at −15°C for 3 hr to initiate crystallization.
8. Thaw samples at 25°C and collect crystals by centrifugation in a tabletop centrifuge at room temperature.
9. Recrystallize both fractions by incubating in 0.1% HCl at 25°C for 4 to 5 hr to collect either betanin or betanidin·HCl.
10. Dissolve each fraction in water and adjust to known volume to give an absorbance reading between 4 and 5 AU.
11. Calculate the concentration using the light absorption at 538 nm and the respective $A^{1\%}$ for betanin and betanidin (i.e., 1120 and 1275, respectively).

See Wyler and Dreiding (1959), Wilcox et al. (1965), and Schwartz and von Elbe (1980) for more information.

Prepare standard curve

12. Separately chromatograph fraction A and C (Fig. F3.1.1), each in multiple concentrations (i.e., between 0.3 and 0.8 AU). Plot concentration versus peak area to obtain a standard curve for betanin and betanidin, respectively.

The standard curves can be used to quantify the respective isomer, since the maximum absorption peaks for the isomers are the same. It is important to use the same conditions (isocratic or gradient elution) for the sample and the standard curve. Results may be expressed in terms of betanin or betanidin, or may be added and reported in terms of total betacyanins.

Analyze sample

13. Chromatograph sample by identical analytical HPLC and determine concentration by comparison with standard curves.

Sources of samples for betacyanin analysis will vary; however, in all cases the pigments are easily extracted with water. Before chromatography, it is recommended that the extracts be filtered using a 0.45-µm HA filter. Use chromatographic conditions as described under initialize HPLC gradient (step 1).

SUPPORT PROTOCOL

EXTRACTION OF BETALAINS FROM BEETS

The analysis of betalains presently has been largely restricted to the determination of the pigment content in beet tissue or to pigment degradation studies in ideal solutions. In the case of beet tissue, the pigments are easily extracted with water, since all betalains are water soluble. This support protocol gives a method to obtain a beet extract from beet tissue. Other food samples which contain betalain can most likely be treated similarly, but interfering substances may prevent the use of the spectrophotometric method. See Critical Parameters for discussion of interfering substances.

Materials

Beet tissue
Celite (Aldrich)
Nitrogen gas (recommended)
Blender

1. Extract 50 g beet tissue with 150 ml distilled water in a blender for 1 min.
2. Mix beet puree with an equal amount of filter aid (celite), quantitatively transfer to a Buchner funnel, and filter through Whatman no. 1 filter paper using reduced pressure.
3. Wash the filter cake several times with distilled water until the extract is colorless.

   The extraction should be carried out under a nitrogen atmosphere to prevent the degradation of the pigments. Flushing the Buchner funnel with nitrogen gas is recommended.

4. Combine all extracts and make to known volume.

   Samples should be analyzed immediately, but can be stored for up to 24 hr under refrigeration if necessary. Longer storage is not recommended.

COMMENTARY

Background Information

Quantitative analysis is used to establish the total pigment content of a colorant and to determine its color strength. The relatively rapid method for the quantification of total pigment content has been very useful in the development of new beet cultivars suitable for pigment production. Present beet cultivars used in vegetable production are relatively low in total pigment. Through a selective breeding process, total pigment per gram of beet tissue has been increased several fold (Goldman et al., 1996). In the selection process it is essential to be able to rapidly determine the total betalain content as well as the betacyanin and betaxanthin content.

Critical Parameters

The multiple component system using beet juice is illustrated in Figure F3.1.2. In Figure F3.1.2 are shown four visible absorption spectra of (1) vulgaxanthin-I, (2) betanin, (3) a mixture of pure betanin and vulgaxanthin-I, and (4) beet juice. The absorption maximum for betanin is 538 nm and 476 nm for vulgaxanthin-I. Betanin also absorbs light at 476 nm and therefore will contribute to the absorption value at that wavelength. Thus, it is necessary to make a correction and subtract from the measured absorption at 476 nm the amount contributed by the presence of betanin to obtain an accurate absorption measurement for vulgaxanthin I. Since the absorption of betanin at 476 nm is not constant and varies with concentration, the ratio A_{476}/A_{538} is used in the calculations. The betanin content can be estimated directly from the absorption measurement at 538 nm, after correcting for the absorption of the impurities at 600 nm. Vulgaxanthin I does not absorb at 538 nm, and therefore no correction is needed for it at this wavelength.

A calculation of the absorption of each pigment in beet juice can be determined with the following equation set:

$$x = a - z$$
$$y = b - z - x/(A_{538}/A_{476})$$
$$z = c - x/(A_{538}/A_{600})$$

For definition of terms see the working equation set (see Basic Protocol 1, step 5).

The quotient A538/A476 for betanin is not influenced by concentration and has a mean value of 3.13 ± 0.03 in phosphate buffer at pH 6.5 over the range 0.2 to 0.8 AU; therefore, in calculations, it is rounded to 3.1. The quotient A538/A600 was found to decrease with increasing light absorption. If the sample is diluted so that the absorption at 538 nm is between 0.4 and 0.5 AU, a value for A538/A600 of 11.5 can be applied. Even if the absorption is as low as 0.2, the error when using 11.5 is within one one-thousandth of a unit. With the two quotients, the equation set above can be simplified to equation set described in the first protocol (see Basic Protocol 1, step 5). The 0.4 to 0.5 AU range is recommended to measure the light absorption, because in this range, the light absorption ratio A538/A600 is 11.5 and in this range solutions obeying Beer's law have the lowest relative error (Bauman, 1962; von Elbe and Schwartz, 1984). pH 6.5 is used because the spectra of pure pigment solutions (Fig. F3.1.1) were determined using this value. Other pH values can be used, but corrections for changes in the light absorption ratios must be made. Similarly, the relative error of readings between 0.09 and 0.7 AU can result in reasonable accuracy.

Beet juice or beet extracts are known to contain other pigments (isobetanin, prebetanin, vulgaxanthin-II) besides betanin and vulgaxanthin-I. The error that is introduced by calculating all betacyanins in terms of betanin and all be-

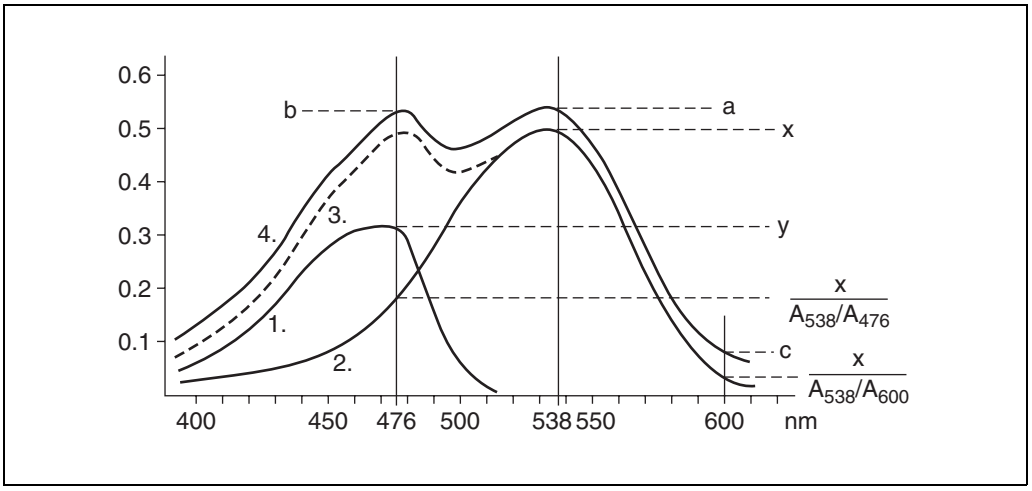

Figure F3.1.2 Calculation of betanin and vulgaxanthin-I in beet juice. Spectrum 1: vulgaxanthin-I; spectrum 2: betanin; spectrum 3: pure betanin plus vulgaxanthin-I; and spectrum 4: beet juice.

taxanthins in terms of vulgaxanthin-I is negligible, as betanin and vulgaxanthin-I comprise ~95% of the pigment concentration and the other betacyanins and betaxanthins have a maximum light absorption similar or very closely to the absorption of betanin and vulgaxanthin-I.

Anticipated Results

The spectrophotometric determination (see Basic Protocol 1) is applicable to fresh beet juice or beet tissue extracts (see Support Protocol). If samples have been exposed to conditions that could result in pigment degradation, caution must be exercised because the corrections made in the method for the presence of impurities may not be sufficient to account for the degradation products (von Elbe et al., 1983).

The results are calculated in terms of betanin, the major betacyanin, and vulgaxanthin-I, the major betaxanthin in beets. Although the minor pigment components also contribute to the light absorption at A_{538} and A_{476} expressing their concentration in terms of betanin and vulgaxanthin-I results in a small or negligible error because of the similarity of their light absorption characteristics to the major pigment components.

The high-performance liquid chromatography method (see Basic Protocol 2) allows for the separation of individual betanin and its aglycon betanidin and their isomers. The aglycon is naturally occurring in beet tissue, but in relatively small amounts. The isomers, isobetanidin and isobetanin, are easily formed when solutions of beet extract are subjected to either heat or acid (Schwartz and von Elbe, 1983). The results can be expressed in terms of the individual pigments or as total betacyanins. The HPLC separates the pigments from degradation products, making it the preferred method over the spectrophotmetric method when substantial amounts of degradation products are believed to be present.

Time Considerations

When juice samples are analyzed for total pigment content (see Basic Protocol 1), the time required per sample involves obtaining appropriate dilutions and a light absorption spectrum and should be <30 min. When tissue extraction is involved, the time required for extraction will be a minimum of 15 min.

When performing HPLC (see Basic Protocol 2) the time of analysis will depend on the conditions used—i.e., isocratic versus gradient. Isocratic separation of the individual betacyanins, as shown in Figure F3.1.2, requires 20 min, compared to 9 min using a gradient elution system.

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Spectrophotometric and Reflectance Measurements of Pigments of Cooked and Cured Meats

Color and color uniformity are important criteria for retail acceptance of both fresh and processed meats. This unit describes the methods for detection and quantitation of pigments in nitrite-cured or uncured cooked meats. Protocols for measurement of fresh meat pigments are presented in *UNIT F3.3.*

The pink cured meat pigment mononitrosylhemochrome is a complex of nitric oxide (NO), ferrous heme iron, and heat-denatured globin protein (Table F3.2.1). The pink nitrosylheme (NO-heme) moiety may be extracted from the protein in aqueous acetone and quantitated by A_{540} (see Basic Protocol 1). The percent nitrosylation may be determined from measurement of ppm NO-heme relative to ppm total acid hematin (hemin) extracted in acidified acetone (see Basic Protocol 2), since NO-heme is completely oxidized to hemin in acid solution (i.e., 1 ppm NO-heme = 1 ppm hemin).

Non-nitrosyl pink pigments, the denatured globin hemochromes, may be present in cooked meats under anaerobic (reducing) conditions, as occurs in the center of large roasts during refrigerated storage. Since the pigments consist of reduced heme iron in association with nitrogenous ligands of denatured proteins, the complex is not extractable for quantitation. Globin hemochromes may be detected, however, by their characteristic dual reflectance maxima near 528 to 530 nm and 558 nm (see Basic Protocol 3).

MEASUREMENT OF NITROSYLHEME CONCENTRATION

BASIC PROTOCOL 1

The measurement of cured meat pigment concentration is based on the A_{540} of the nitrosyliron(II)protoporphyrin group (also known as nitrosylheme or NO-heme; mol. wt. 646) in an extraction solution of 80% (final) acetone in water, taking into consideration the 70% water content of the meat sample. Hornsey (1956) established that only the pink NO-heme was extracted in 80% acetone. Heme groups from fresh meat pigments (Table F3.2.1) are not extractable in 80% acetone. However, upon acidification with hydrochloric acid, NO-heme in 80% acetone was completely oxidized to hemin. Thus, NO-heme concentrations could be expressed in equivalent ppm hemin.

NOTE: Ferroprotoporphyrin is the term used for reduced heme (mol. wt. 616; Windholz et al., 1976). Hematin (mol. wt. 633) is one of several terms used for the oxidized or ferric heme. Other terms include ferriporphyrin hydroxide or ferriheme hydroxide, due to the binding of a hydroxyl to the ferric heme iron. Hornsey (1956) used the term "acid hematin" (mol. wt. 652) to describe the heme oxidation product in acidified acetone solutions. The more common term "hemin" is used in this unit. It is also known as chlorohemin, due to binding of a chloride ion to the ferric heme iron.

Materials

Meat sample to be tested
Aqueous acetone (see recipe)

15 × 90–mm glass test tubes with screw caps
Glass stirring rod
50-mm-diameter funnels
Whatman no. 42 filter paper, 9-cm diameter
Spectrophotometer with halogen lamp and 1-cm-pathlength quartz cuvettes

Contributed by Daren Cornforth
Current Protocols in Food Analytical Chemistry (2001) F3.2.1-F3.2.8

Table F3.2.1 Major Pigments of Fresh Cooked, and Cured Meats[a]

Pigment	Formation	Heme status	Heme iron state	Globin status	Color
Fresh meat:					
Myoglobin	Deoxygenation of oxymyoglobin; reduction of metmyoglobin	Intact	Fe(II)	Native	Purplish red
Oxymyoglobin	Oxygenation of myoglobin	Intact	Fe(II)	Native	Bright red
Metmyoglobin	Oxidation of myoglobin	Intact	Fe(III)	Native	Brown
Cooked meat:					
Denatured globin hemochrome	Heat denaturation of myoglobin; reduction of globin hemichrome	Intact	Fe(II)	Denatured	Pink or red
Denatured globin hemichrome	Heat denaturation of metmyoglobin; oxidation of globin hemochrome	Intact	Fe(III)	Denatured	Gray or brown
Cured meat:					
Nitrosylmyoglobin (NO-Mb)	Nitric oxide complex with myoglobin	Intact	Fe(II)	Native	Red
Mononitrosyl-hemochrome	Heating of NO-Mb	Intact	Fe(II)	Denatured	Bright red or pink
Mononitrosyl-hemichrome	Oxidation of mononitrosyl hemochrome (fading in air)	Intact	Fe(III)	Denatured	Gray or brown
Verdoheme	Excess nitrite oxidizes porphyrin ring	Ring opened	Fe(III)	Absent	Green

[a]Adapted from von Elbe and Schwartz (1996) courtesy of Marcel Dekker, Inc.

NOTE: The following operations should be conducted in subdued light to reduce pigment fading during extraction.

1. Trim oxidized (faded brown or gray) surfaces of a meat sample and dice a lean portion into 2- to 3-mm cubes.
2. Weigh out duplicate 2.0 g samples and transfer to 15 × 90–mm glass test tubes containing 9.0 ml aqueous acetone.

   This 90% aqueous acetone solution should yield a final of 80% acetone and 20% water when the 70% water content of the sample is taken into account. If the sample water content deviates from 70%, the solution should be adjusted.
3. Macerate the meat mass thoroughly with a glass stirring rod (~1 min). Seal tube caps to reduce evaporation.
4. Hold at room temperature in subdued light for 10 min, then filter through Whatman no. 42 filter paper (9 cm diameter) into a clean test tube. Cap the tube to reduce evaporation.
5. Transfer filtrate to a 1-cm-pathlength quartz cuvette and read A_{540} within 1 hr against a blank cuvette containing aqueous acetone.

   Disposable plastic cuvettes should not be used, as the acetone will solubilize the cuvette and cause turbidity in the reference cell.

6. Calculate the concentration of NO-heme in accordance with the relationship NO-heme concentration (ppm hemin) = $A_{540} \times 289$ ppm.

This equation is derived from the equation $A_{540} = abC$, where A_{540} is sample absorbance, a is absorptivity, b is length of light path (1 cm), and C is concentration of absorbing material (in mM). Absorptivity is a constant dependent upon the wavelength of radiation and the nature and molecular weight of the absorbing material. Millimolar absorptivity, denoted by the symbol E, is the product of absorptivity and molecular weight, with units of liter $mmol^{-1}$ cm^{-1}. The E^{mM}_{540} of NO-heme in 80% aqueous acetone is 11.3 liter $mmol^{-1}$ cm^{-1} (Hornsey, 1956).

The conversion factors needed to express the concentration in ppm hemin (1 ppm = 1 μg/g) and the dilution factor must also be taken into consideration. The dilution factor is the total extraction fluid volume (ml) divided by the sample weight (g). The total extraction fluid volume includes the water content of the sample plus the amount of aqueous acetone solution. Most cooked meats have ~70% water. Thus, for a 2 g sample containing 1.4 ml water, the total extraction volume = 1.4 ml + 9 ml acetone solution, and the dilution factor = 10.4 ml/2 g sample = 5.2. Pearson and Tauber (1984) rounded the dilution factor to 5, but for best results the decimal should be retained. Also, if the sample water content is significantly different from 70%, the dilution factor should be recalculated as appropriate.

Thus, sample NO-heme concentration (ppm) = $A_{540} \times$ (1 cm mmol NO-heme/11.3 liter) × 1/cm × total extraction volume in ml/sample weight in g × 1 mol hemin/mol NO-heme × 652 g hemin/mol hemin × 10^6 μg/g × 10^{-3} mol/mmol × 10^{-3} liter/ml. Substituting the dilution factor of 5 into this equation and simplifying, NO-heme concentration = $A_{540}/11.3 \times 5 \times 652$ μg/g = $A_{540} \times 289$ ppm (as hemin). Hornsey (1956) and Pearson and Tauber (1984) rounded the concentration factor to 290 ppm.

MEASUREMENT OF TOTAL HEME CONCENTRATION

BASIC PROTOCOL 2

Total heme pigments in meat samples are determined after extraction with acidified acetone solution, since the heme groups of both fresh meat pigments (myoglobin, oxymyoglobin, and metmyoglobin; Table F3.2.1) and cured meat pigment (nitrosylhemochrome; Table F3.2.1) are solubilized and oxidized to hemin (Hornsey, 1956). Hemin (acid hematin; mol. wt. 652) is quantitated by its absorption peak at 640 nm. After determination of NO-heme concentration (Basic Protocol 1) and total heme (as hemin; this protocol) on the same sample, the efficiency of meat curing may be expressed as a percentage: curing efficiency (%) = (ppm NO-heme/ppm total heme) × 100. Good or acceptable pigment conversion is generally considered to be 80% to 90% of the heme pigments converted to nitrosylheme (Pearson and Tauber, 1984).

Materials

Meat sample to be tested
Acidified acetone (see recipe)

15 × 90–mm glass test tubes with screw caps
Glass stirring rod
50-mm-diameter funnels
Whatman no. 42 filter paper, 9-cm diameter
Spectrophotometer with halogen lamp and 1-cm-pathlength quartz cuvettes

NOTE: The following operations should be conducted in subdued light to reduce pigment fading during extraction.

1. Trim oxidized (faded brown or gray) surfaces of a meat sample and dice a lean portion into 2- to 3-mm cubes.

2. Weigh out duplicate 2.0 g samples and transfer to 15 × 90–mm glass test tubes containing 9.0 ml acidified acetone. To minimize acetone evaporation, use a cali-

brated pipet rather than a graduated cylinder to transfer acetone solution into glass test tubes.

This acidified 90% acetone solution should yield a final of 80% acetone and 20% water when the 70% water content of the sample is taken into account. If the sample water content deviates from 70%, the solution should be adjusted.

3. Macerate the meat mass thoroughly with a glass stirring rod (~1 min). Seal tube caps to reduce evaporation.
4. Hold at room temperature in subdued light for 1 hr, then filter through Whatman no. 42 filter paper (9 cm diameter) into a clean test tube. Cap the tube to reduce evaporation.
5. Transfer filtrate into a 1-cm-pathlength quartz cuvette and read A_{640} within 1 hr against a blank cuvette containing acidified acetone.

   Disposable plastic cuvettes should not be used, since the acetone will solubilize the cuvette and cause turbidity in the reference cell.

6. Calculate total hemin concentration using the equation total hemin concentration = $A_{640} \times 680$ ppm.

   This equation is derived as described for NO-heme concentration (see Basic Protocol 1, step 6). Millimolar absorptivity, E^{mM}_{540}, of hemin in 80% acetone is 4.8 liter mmol^{-1} cm^{-1} (Hornsey, 1956), giving the constant 680 in place of 289.

BASIC PROTOCOL 3

REFLECTANCE DETECTION OF GLOBIN HEMOCHROMES

The term globin hemochrome or denatured globin hemochrome (Table F3.2.1) is used to describe the heterogeneous mixture of pink pigments present under reducing conditions in uncured cooked meats. Globin hemochromes are pink complexes between ferrous heme iron of heat-denatured myoglobin and various nitrogenous compounds in meat, possibly including amino acids, nicotinamide, or nitrogenous side chains of other heat-denatured proteins. Since heat denaturation of proteins is necessary for their formation, they often occur in well-cooked meats (internal temperature >76°C). Pink color may intensify during anaerobic storage after cooking, as the brown hemichromes are slowly reduced. The globin hemochromes are detected in meat slices by their characteristic reflectance maxima near 528 and 558 nm.

Materials

White standard (powdered barium sulfate)
Meat sample to be tested

Recording spectrophotometer with integrating sphere attachment, with ports for sample and standard
Clear polyethylene vacuum bags (1.5-mil thickness)

1. Standardize a recording spectrophotometer to 100% reflectance from 420 to 700 nm, using the white standard (powdered barium sulfate) in both the sample and standard ports of the reflectance attachment.
2. Obtain a uniform meat slice that is 3 cm × 3 cm (sufficient to completely cover the sample port on the reflectance attachment) and >3 mm thick.
3. To exclude air and minimize fading, rapidly place the fresh slice in a clear polyethylene vacuum bag (1.5-mil thickness). Press the bag against the sample from bottom to top to remove air bubbles.

The sample remains in the clear polyethylene bag during reflectance measurement to prevent fading.

4. Place the bagged sample snugly in the sample port of the reflectance sphere, with the freshly sliced surface facing inward (toward the detector). Record reflectance (% of standard) from 420 to 700 nm.
5. Allow a control meat slice to fade in air for 15 to 30 min.
6. Bag the sample and record the reflectance spectra as described for fresh samples.
7. Subtract baseline spectrum (control slice) from sample spectrum obtained in step 4.

REAGENTS AND SOLUTIONS

Use distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Aqueous acetone

Add 18 ml distilled water to a 200-ml volumetric flask. Add spectrophotometric grade acetone, mix, and bring to volume with additional acetone. Store up to 6 months at 3°C.

Acidified acetone

To 4 ml concentrated hydrochloric acid, add distilled water to a total volume of 20 ml and mix. Transfer dilute hydrochloric acid to a 200-ml volumetric flask. Add spectrophotometric grade acetone, mix, and bring to volume with additional acetone. Store up to 1 month at 3°C. Discard solutions that become yellowish.

CAUTION: *For safety reasons, concentrated acids are usually added slowly to a larger volume of water. The reader may wish to modify this recipe so that the acid is added to the water.*

COMMENTARY

Background Information

Nitrosylheme, also known as NO-heme or nitrosyliron(II) protoporphyrin, is readily extracted from the cured meat pigment mononitrosylhemochrome (Killday et al., 1988) into an aqueous acetone solution for quantitation by spectroscopy. Hornsey (1956) established the following important points for accurate NO-heme quantitation.

1. Maximum NO-heme extraction occurred with an acetone/water ratio of 75% to 85% (Table F3.2.2).
2. Only the nitrosylheme derivative of the muscle pigments was extracted in 80% acetone. Heme from metmyoglobin, oxymyoglobin, or deoxymyoglobin (or hemoglobin) was not extracted.
3. The absorption spectra for extracted NO-heme exhibited a broad peak from 535 to 565 nm. Absorbance from 680 to 700 nm was <0.03.
4. The NO-heme extract from uncooked cured meat or model solutions with pure hemoglobin or myoglobin faded rapidly.
5. Extracts from cooked cured meat were stable for ≥1 hr, indicating that reducing substances (probably cysteine or glutathione) were also extracted, conferring stability against light-catalyzed air oxidation. (In uncooked cured meat, these compounds would also be present but at least partly oxidized, depending on the degree of oxygen incorporation during maceration and other processing steps.)
6. Fading of acetone extracts of pure NO-myoglobin solutions could be prevented by adding 1 ml of fresh 0.5% neutralized cysteine hydrochloride to 9 ml of NO-myoglobin solution.

Hornsey (1956) also found that extraction with an acidified 80% acetone solution for 1 hr gave a hemin solution, derived from oxidation of the heme moiety of both NO-heme and non-nitrosylated heme pigments. Hemin absorption spectra exhibited distinct peaks at 512 and 640 nm. Hornsey (1956) used the A_{640} of sample filtrates as a measure of the total heme pigments. Solutions of both hemin and NO-heme in 80% acetone conformed with Beer's

Table F3.2.2 Effect of Acetone Concentration on Optical Density (A_{540}) of Extracts from Lean, Cooked, Cured Pork Ham[a]

Acetone concentration (%)	A_{540}
96.5	0.324
93	0.330
86	0.360
80	0.370
75	0.360
70	0.308
65	0.270
60	0.225

[a]Adapted from Hornsey (1956) with permission from John Wiley & Sons on behalf of the Society of Chemical Industry.

Law, with straight lines passing through the origin.

In the Hornsey (1956) procedure, the sample was minced thoroughly, and a 10 g sample was mixed in a tall beaker (to prevent undue evaporation) with 10 ml of a solution of 40 ml acetone and 3 ml water. Total fluid volume was 50 ml, including the 7 ml water in the sample. This procedure was preferred over dilution of sample to 50 ml final volume, since calculations were simplified and correction for the volume of insoluble meat tissues was avoided. Later workers have modified the procedure, using smaller samples and less solvent. Pearson and Tauber (1984) used a 2 g sample and capped test tubes to prevent acetone evaporation, and Carpenter and Clark (1995) used 5 g samples.

The Hornsey (1956) procedure and its modifications have received widespread acceptance as relatively rapid measures of the adequacy of cure development in processed meats. The Hornsey procedure is also an accurate method for nutritional assessment of heme and heme iron content of meats (Carpenter and Clark, 1995), where ppm heme iron = ppm total heme/11.7. However, one caveat should be noted. The total heme pigment measurement is higher in cured meats than in similar uncured samples. Roasted turkey breast meat, for example, was reported by Ahn and Maurer (1989a) to have 23, 26, 34, and 34 ppm total pigment in samples formulated with 0, 1, 10, and 50 ppm nitrite, respectively. This effect should be considered to avoid overestimation of the heme iron content of cured meats.

Tappel (1957) was among the first to record spectral characteristics of the pink pigments of well-cooked meats, noting reflectance minima at 424, 528 to 530, and 555 to 560 nm for denatured globin hemochrome, and 423, 523 to 525, and 555 to 558 nm for nicotinamide hemochrome. The term globin hemochrome is used to describe the heterogeneous mixture of pink pigments occurring under reducing conditions in uncured cooked meats. Globin hemochromes are pink complexes between ferrous heme iron of heat-denatured myoglobin and various nitrogenous compounds in meat, possibly including amino acids, nicotinamide, or nitrogenous side chains of other heat-denatured proteins. Since heat denaturation of proteins is necessary for their formation, they often occur in well-cooked meats (internal temperature >76°C; Ghorpade and Cornforth, 1993). Pink color may intensify during anaerobic storage after cooking, as the brown hemichromes are slowly reduced. Tappel (1957) and Tarladgis (1962) used sodium dithionite to rapidly reduce the globin hemichromes of cooked meat slices to pink globin hemochromes for spectroscopic studies. Tappel (1957) also exposed dithionite-reduced slices to carbon monoxide, and noted reflectance minima at 542 and 570 nm for denatured globin carbon monoxide hemochrome. Reviewers including this author (Cornforth et al., 1991) have attributed the surface pinking of meats heated in a gas oven in part to the presence of carbon monoxide. However, more recent studies (Cornforth et al, 1998) indicate that nitrogen dioxide is the combustion gas responsible for surface pinking. Although carbon monoxide binds to myoglobin in fresh meats, it is released upon cooking (Watts et al., 1978). Carbon monoxide contrib-

utes to pinking in cooked meats only under anaerobic or reducing conditions, unusual at the surface of meat cooked in a gas oven.

Cytochrome *c* is more heat stable than myoglobin, and may contribute to residual pinking in cooked pork or poultry (Ahn and Maurer, 1989b; Girard et al., 1990). Cytochrome *c* solutions exhibit absorption maxima at 414, 520, and 550 nm. Cytochrome *c* is more resistant than other pink pigments to fading upon exposure of the meat surface to air (Girard et al., 1990). Pinkness of uncured cooked meat slices that fades rapidly after slicing is likely due to presence of globin hemochromes.

Critical Parameters and Troubleshooting

Incomplete pigment extraction (NO-heme and total pigments)

Incomplete pigment extraction may be due to insufficient maceration. If so, dice or grind meat into smaller particles, macerate more thoroughly with the glass rod, or homogenize the sample for 20 to 30 sec with a probe-type blender (e.g., Kinematica polytron with small head).

Incomplete pigment extraction may also be due to variable sample moisture content. Maximum pigment extraction is obtained in an extraction solution of 80% acetone and 20% water, including the water content of the sample. The procedure as outlined is for samples with 70% ± 3% water. Determine the water content of the sample, and then adjust the water content of the extraction solution as needed to obtain a final 80% acetone and 20% water in the extraction (Carpenter and Clark, 1995).

Excessive evaporation of acetone during extraction will alter the acetone/water ratio, causing incomplete pigment extraction. The tubes should be capped during extraction and handling to minimize evaporation.

Fading (NO-heme and total pigments)

Extracted samples may fade if exposed to bright light or if held for an excessive period before obtaining absorbance values. Keep sample in subdued light during extraction, and obtain absorbance values within 1 hr. Uncooked cured meat extracts or extracts of pure pigments in model systems are more prone to fading than extracts from cooked meat systems. Addition of 0.2 ml fresh cysteine solution (0.5% cysteine HCl) per 10 ml total fluid (acetone plus water plus cysteine solution) will prevent fading for several hours.

Fading (globin hemochromes)

Pink globin hemochromes tend to fade rapidly after slicing of cooked meats. Fading may be slowed by covering the exposed surface with a transparent plastic film (e.g., Saran wrap or polyethylene) to retard pigment oxidation by atmospheric oxygen, allowing more time to obtain a representative sample reflectance spectrum. Spreading a thin coating of vegetable or mineral oil over cut surfaces may accomplish the same purpose, as it has been observed that pink pigments from roasted pork are resistant to fading when the lean portion was covered by melted fat.

Some investigators have soaked cooked meat slices in freshly prepared 1% sodium dithionite solution to maintain the pigments in the reduced state. However, this is not truly representative of the fresh meat slice, since dithionite will also reduce the brown globin hemichromes, intensifying the pink color to a level greater than that actually observed after slicing.

Anticipated Results

Total heme pigments vary among species and muscles, with levels >140 ppm for cooked beef products (Pearson and Tauber, 1984). Carpenter and Clark (1995) used the acetone extraction method of Hornsey (1956) to determine heme iron content of various cooked meats. They reported heme iron levels of 21, 9, 2.2, and 1.4 ppm for cooked beef round, pork picnic, pork loin, and chicken breast, respectively. Hemin (mol. wt. 652) is 8.54% iron. Thus, these meats contained 245, 105, 25, and 16 ppm total heme, respectively (Carpenter and Clark, 1995). Ahn and Maurer (1989a) reported a value of 23 ppm total heme in cooked turkey breast.

About 80% to 90% conversion of heme pigments to nitrosylhemochrome is desirable for cured meats (Pearson and Tauber, 1984). However, beef pastrami with typical cured color may have as low as 62% conversion (94 ppm NO-heme out of 153 ppm total heme; Cornforth et al., 1998).

Globin hemochrome levels in cooked meats have not been quantitated. Their presence is indicated, however, by a reflectance spectrum with a large reflectance minimum at ~558 nm and a smaller minimum or shoulder at ~528 to 530 nm (Tappel, 1957; Ghorpade and Cornforth, 1993).

Time Considerations

Preparation of aqueous and acidified acetone solutions for NO-heme and total heme determination takes 30 min. Measurement of NO-heme concentration of a single sample takes an additional 30 min. Measurement of total heme concentration of a single sample takes 1.5 hr. Often it is desirable to make both measurements on the same sample. By starting the 1-hr extraction for total heme and then the 10-min extraction for NO-heme, both determinations may be done in 2 hr (including the 30 min for preparation of solutions).

Approximately 30 min is sufficient to obtain a reflectance spectrum for detection of globin hemochromes. Approximately 1 hr is required to install the reflectance attachment and run calibration curves.

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Key References

Hornsey, 1956. See above.

This paper provides the basic information for spectrophotometric determination of nitrosyl and total heme pigment levels in cured meats.

Killday et al., 1988. See above.

Mass spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and infrared spectroscopy indicated that the cured meat pigment was mononitrosylhemochrome. Contrary to previous reports, no evidence was found to indicate presence of dinitrosylheme complexes.

Pearson and Tauber, 1984. See above.

This paper is an adaptation of the Hornsey (1956) method for measurement of nitrosyl and total heme pigments in cured meats, using 2 g meat samples rather than 10 g samples. Thus, less reagent is needed and more samples may be analyzed at the same time.

Contributed by Daren Cornforth
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The author thanks Dr. Charles E. Carpenter (Nutrition & Food Sciences, Utah State University, Logan, Utah) for time and effort spent proofreading this contribution.

Measurement of Discoloration in Fresh Meat

The discoloration of fresh meat is an important process, which is determined by the relative concentration of the three redox forms of myoglobin (deoxymyoglobin, oxymyoglobin, and metmyoglobin). Loss of the desirable cherry-red appearance with subsequent replacement by reddish browns and browns is a natural process affected by a variety of intrinsic and extrinsic parameters. Because consumers often use meat color as a basis for product selection or rejection, the loss in economic value that accompanies fresh meat discoloration can be substantial. Considerable research by the meat industry and Agricultural Experiment Stations has focused on enhancing the maintenance of desirable fresh meat color through a variety of processing and packaging techniques. In order to evaluate the efficacy of these procedures, it is critical that appropriate methods are used to objectively measure and describe changes in fresh meat color (AMSA, 1991).

In general, the approaches for measuring fresh meat discoloration are relatively straightforward and simple. Fresh meat may be merchandised in either minced (ground meat) or whole (e.g., steaks, roasts) forms. While the principles involved in objectively measuring changes in color are the same for both product types, sample preparation and instrumentation are different. Minced products are often homogenized and the pigment-containing extract is analyzed by transmission or absorbance spectrophotometry (see Basic Protocol 1). Although this is the method of choice for myoglobin and hemoglobin quantification, it does not offer an accurate representation of surface color as observed by the consumer. Whole-muscle products are analyzed with reflectance techniques or colorimetry (see Basic Protocol 2 and Alternate Protocol, respectively). With these rapid methods, repeated measurements may be recorded from the same sample over time. Fresh meat discoloration projects are generally carried out for 3 to 7 days, with color analysis being performed either every day or every other day. The time course for frozen samples is longer (e.g., 90 days), with color analysis being performed much less often (e.g., every 30 days). It is imperative to establish sampling parameters based on specific research objectives prior to the beginning of a study. Finally, many investigators find it necessary to utilize a model approach for studying the biochemistry of myoglobin in vitro. These experiments often require the isolation (see Basic Protocol 3) and redox manipulation (see Basic Protocol 4) of myoglobin.

ANALYSIS OF METMYOGLOBIN IN GROUND MEAT EXTRACTS

BASIC PROTOCOL 1

Ground meat generally discolors much faster than whole-muscle cuts. The mincing of meat destroys cellular integrity and liberates a variety of prooxidants, which can accelerate the discoloration process. In addition, iron from meat grinding equipment surfaces can become incorporated into the meat and serve as an oxidation catalyst (Faustman et al., 1992). The penetration of oxygen, a necessary component for prooxidant reactions, into ground meat products is also greater than in whole-muscle cuts because of the porous structure. These factors promote the formation of metmyoglobin from either oxy- or deoxymyoglobin. The method of Krzywicki (1982) can be used to quantify the total concentration of myoglobin, as well as the relative concentrations of oxidized, deoxygenated, or oxygenated forms of the pigment using absorbance values at 572, 565, 545, and 525 nm.

Materials

40 mM sodium phosphate buffer, pH 6.8 (*APPENDIX 2A*), ice cold
Ground meat sample
Control sample

Contributed by Cameron Faustman and Amy Phillips
Current Protocols in Food Analytical Chemistry (2001) F3.3.1-F3.3.13

Spectrophotometer able to scan visible spectrum
Blender
Whatman no. 1 filter paper

Establish baseline

1. Turn on a spectrophotometer and allow it to warm up for 30 min prior to use.
2. Pipet 1.0 ml of 40 mM sodium phosphate buffer, pH 6.8, into a disposable cuvette and place it in the sample port of the spectrophotometer.
3. Scan the buffer from 650 nm to 450 nm to establish a baseline.

Prepare samples

4. Select a 25 g sample of ground meat from an appropriate location of the meat product. Additionally, select a proper control sample to prepare in parallel.

   The exact location of the sample (surface versus deep versus surface plus deep) will depend on the experimental design and question being investigated.

   In general, replicate samples are essential when analyzing ground meat color. An efficient practice may involve the preparation of patties (~25 g) so that an entire sample is used during analysis. A small petri dish (~5 cm in diameter) serves as an excellent mold for patty formation while maintaining geometric shape, surface area, and compaction.

   Control, nontreated samples subjected to the same preparation as treated samples must be used to allow for comparisons between treatments.

5. Homogenize sample in a blender with 10 vol ice-cold 40 mM sodium phosphate buffer, pH 6.8, for 45 sec at high speed.
6. Filter homogenate using a Whatman no. 1 filter paper.

   In some situations, filtration through Whatman no. 1 filter paper may not yield a clear solution (e.g., ground meat displayed for >4 days). When this occurs, pass filtrate through a 0.40-μm filter attached to a syringe.

Determine metmyoglobin concentration

7. Pipet 1.0 ml filtrate into a fresh disposable cuvette and place it in the sample port of the spectrophotometer.
8. Scan sample from 650 nm to 450 nm and record absorbance values at 5-nm intervals. In addition, record absorbance value at 572 nm.
9. Estimate total concentration of myoglobin in the sample from the A_{525} value using an extinction coefficient of 7.6 mM^{-1} cm^{-1} and a path length of 1 cm (Bowen, 1949) as follows:

   Myoglobin (mM) = $[A_{525}/(7.6\ mM^{-1}cm^{-1} \times 1\ cm)]$.

10. Calculate the relative concentration of metmyoglobin (MetMb) using the following equation:

    $\%\ \text{MetMb} = -2.541R_1 + 0.777R_2 + 0.800R_3 + 1.098$

    where R_1 is A_{572}/A_{525}, R_2 is A_{565}/A_{525}, and R_3 is A_{545}/A_{525} (Krzywicki, 1982).

    In general, percent metmyoglobin is reported and not percent deoxy- or percent oxymyoglobin. Formulas for the two ferrous myoglobin forms can be obtained from Krzywicki (1982).

ANALYSIS OF METMYOGLOBIN IN FRESH MEAT SURFACES USING DIFFUSE REFLECTANCE SPECTROPHOTOMETRY

The surfaces of whole muscle cuts can be analyzed using diffuse reflectance spectrophotometry, a nondestructive method that is closely related to visual assessment of meat discoloration. Unlike analysis of ground meat extracts, surface analysis allows samples to be easily repackaged and stored for future use. In addition, appropriate control samples must be utilized so that valid comparisons may be made between treatments. In the following procedure, Stewart et al. (1965) used the Kubelka-Munk equation (Judd and Wyszecki, 1963) to relate both the absorption (K) and scattering (S) of light by a meat sample at a given wavelength. The K/S ratios from different wavelengths can then be manipulated to estimate the relative presence of metmyoglobin on the meat surface.

Materials

Meat sample
Control sample

Spectrophotometer able to scan visible spectrum and equipped with integrating sphere
Barium sulfate plates (to standardize spectrophotometer)
Fresh meat PVC film

Establish baseline

1. Attach an integrating sphere to a spectrophotometer, turn on the spectrophotometer, and allow it to warm up for 30 min prior to use.
2. Place a barium sulfate plate wrapped in a single layer of fresh meat PVC film in both the reference and sample ports.
3. Establish a baseline by scanning from 650 nm to 450 nm.

   Note that the slit width for diffuse reflectance spectrophotometry must be sufficiently wide to avoid excessive noise, which will result from surface measurements. The exact slit width will depend on the instrument, but 5 nm is routinely used.

Analyze samples

4. Select an appropriately sized meat sample (at least 1 cm thick) and wrap tightly with a single layer of fresh meat PVC film. Additionally, select a proper control sample to prepare in parallel.

   Wrapping the sample will prevent contamination of the inner white surface of the integrating sphere with any meat exudate.

   Control, nontreated samples subjected to the same preparation as treated samples must be used to allow for comparisons between treatments.

5. Remove barium sulfate plate and place sample in the sample port of the spectrophotometer.
6. Scan sample from 650 nm to 450 nm and record absorbance values at 5-nm intervals. In addition, record absorbance value at 572 nm.

   If research objectives require analysis over time, repackage and store samples appropriately after recording absorbance values.

Calculate percent metmyoglobin

7. Convert absorbance to reflectance using the following equation:

$$R_a = 2 - \log R_\lambda$$

where R_a is reflectance expressed as an absorbance value (as measured in step 6) and R_λ is the calculated percent reflectance.

8. Calculate the K/S_λ ratio using the following equation: $K/S_\lambda = (1 - R_\lambda)^2/(2 \times R_\lambda)$, where K is the absorption coefficient, S_λ is the scattering coefficient at a given wavelength, and R_λ is the reflectance at a given wavelength.

9. Calculate the K/S value using the following equation: $K/S = (K/S_{572})/(K/S_{525})$, where K/S_λ is the value obtained from step 8.

   Stewart et al. (1965) recognized a large difference in light absorption at 572 nm between metmyoglobin and ferrous myoglobins. They proposed a procedure by which the K/S_{572} would be calculated and divided by K/S_{525} (to account for potential differences in muscle myoglobin concentrations that could exist between different carcasses) to yield a K/S value for meat in which metmyoglobin comprised from 0% to 100% of the total pigment. In order to obtain these standards, ground beef was treated with potassium ferricyanide, an oxidizing agent, to obtain 100% metmyoglobin, or with sodium hydrosulfite, a reductant, to obtain 100% ferrous myoglobin (0% metmyoglobin). Approximately 20 samples of each of the two treatments were measured and the K/S plotted against 0% and 100% metmyoglobin. Stewart et al. (1965) assumed a linear relationship between K/S and metmyoglobin concentration. Franke and Solberg (1971) subsequently published results that supported this assumption, although they advocated a modified method for estimating percent metmyoglobin.

10. Calculate percent metmyoglobin (MetMb) using the following equation:

    $\%\ \text{MetMb} = [100 - (K/S - 0.56)]/0.0084$

    where K/S is the value obtained from step 9.

    The equation above was derived by Stewart et al. (1965) and is applicable to many situations. However, a similar equation for percent metmyoglobin may be derived by obtaining several meat samples (4 $cm^2 \times 1$ cm thick) of similar composition to those used in the particular study and subjecting one half of the samples to sodium hydrosulfite and one half to potassium ferricyanide, as follows. To convert meat pigment entirely to reduced myoglobin, dissolve 0.02 g sodium hydrosulfite in 15 ml water and submerge the meat sample in the solution for ~1 min. A typical absorption spectra of reduced myoglobin will persist for at least 25 min. For conversion to metmyoglobin, dissolve 0.1 g potassium ferricyanide in 15 ml water and submerge the meat sample in the solution for ~1 min. A typical metmyoglobin spectrum is produced if this is examined immediately; however, depending on the meat sample, the ferric pigment may be rapidly reduced. Once absorption values are collected, they may be plotted against 0% to 100% metmyoglobin to obtain a straight line curve. Because a linear relationship is assumed between absorption and metmyoglobin concentration, metmyoglobin values may be extrapolated from the graph.

    The relative proportion of metmyoglobin can be determined using one of various procedures. Although the authors prefer the Stewart et al. (1965) method as it provides values that correlate well with visual color assessment, Broumand et al. (1958), Van den Oord and Wesdorp (1971), Strange et al. (1974), and Bevilacqua and Zaritzky (1986) also describe procedures to evaluate meat color using reflectance spectrophotometry. Also, Krzywicki (1979) utilized similar approaches in an attempt to develop equations for calculating the relative proportions of deoxy-, oxy-, and metmyoglobin on beef surfaces. Krzywicki (1979) utilized extinction coefficients for myoglobin in solution, and attempted to account for structural impacts on light scattering by subtracting reflectance values obtained at 730 nm, a wavelength at which the author maintained that myoglobin would demonstrate no absorbance, and thus would control for structural differences between meat samples.

ANALYSIS OF FRESH MEAT SURFACE COLOR USING COLORIMETRY

ALTERNATE PROTOCOL

Depending upon the equipment available for analysis, the surfaces of whole-muscle cuts may also be analyzed using colorimetry as opposed to diffuse reflectance spectrophotometry (see Basic Protocol 2). Because samples are not consumed during analysis, they may simply be repackaged and stored, according to study protocol, for future use. Colorimeters provide L^*, a^*, and b^* values, also referred to as Commission Internationale de l'Éclairage (CIE) Lab values (Clydesdale, 1978). Although L^* and b^* values are not extensively reported in meat discoloration studies, they give an indication of lightness and yellow/blue color, respectively. For the purposes of following fresh meat discoloration, the a^* value, or degree of redness, is the most useful. In general, a^* values will decrease with display time. Mathematical manipulation of the a^* and b^* values can be used to obtain chroma, $[(a^*)^2 + (b^*)^2]^{1/2}$, an indication of the saturation of a color. In addition to chroma, hue angle, $\tan^{-1}(b^*/a^*)$, may be plotted as a function of storage and/or be correlated with sensory assessment of appearance (Clydesdale, 1978).

Materials

Meat sample
Control sample

Colorimeter (e.g., Chromameter CR-200, Minolta)
White (or red/pink) standardization plate (should be provided with colorimeter)
Fresh meat PVC film (optional)

1. Standardize a colorimeter against a white standardization plate or, in some cases, a red/pink plate.

   The white plate is used by most investigators for standardization, but there may be instances where the colored standard is preferred. Whichever is chosen, that same plate must be used throughout the discoloration study. Additionally, the exact specifications of the standardization plate should be indicated in any scientific report.

2. Select an appropriately sized meat sample (at least 1-cm thick) to allow placement within the sample port of the benchtop colorimeter, or to permit placement of the hand-held colorimeter directly on the sample surface. Additionally, select a proper control sample to prepare in parallel.

   Control, nontreated samples, subjected to the same preparation as treated samples, must be used to allow for comparisons between treatments.

3. Wrap sample with fresh meat PVC film if needed.

   With hand-held colorimeters, the authors have found it unnecessary to wrap the meat with film; however, this may be necessary with certain benchtop instruments to prevent harming the equipment. If the meat sample is wrapped in film, the standardization plate should also be wrapped in film.

4. Make and record two to three readings of L^*, a^*, and b^* values per sample surface.

   If research objectives require analysis over time, repackage and store samples appropriately after taking measurements.

5. Average recorded values and calculate chroma or hue angle for the sample.

   An average of two to three readings per sample surface will more accurately reflect meat color.

ISOLATION OF TOTAL MYOGLOBIN FOR IN VITRO STUDIES

In order to study the autoxidation mechanism of myoglobin, one of the main factors involved in meat discoloration, the isolation and purification of myoglobin is necessary. Myoglobin (mol. wt. ~17,000 Da) can be purified readily from skeletal or cardiac muscle of meat-producing animals. It is a robust protein and its red color permits easy visualization of its progress during chromatography. A variety of purification procedures for myoglobins have been published; a straightforward approach adapted from both Wittenberg and Wittenberg (1981) and Trout and Gutzke (1996), which provides substantial yields of myoglobin using relatively inexpensive equipment, is provided.

Materials

Diced beef muscle trimmed of visible fat and connective tissue
Homogenization buffer (10 mM Tris·Cl/1 mM EDTA, pH 8.0), 4°C
Sodium hydroxide
Ammonium sulfate
Dialysis buffer (10 mM Tris·Cl/1 mM EDTA, pH 8.0), 4°C
Chromatography elution buffer (5 mM Tris·Cl/1 mM EDTA, pH 8.5), 4°C

Blender
Cheesecloth
Centrifuge capable of spinning at 20,000 × *g*, 4°C
Dialysis tubing (MWCO 12,000 to 14,000)
Sephacryl S-200 HR chromatography column (30 × 2.5–cm)
Peristaltic pump

Additional reagents and equipment for protein assays (*UNIT B1.1*) or for calculating concentration using extinction coefficients (see Basic Protocol 1)

NOTE: To minimize formation of metmyoglobin, homogenization and all subsequent steps should be performed at low temperature (0° to 5°C) and high pH (8.0 to 8.5).

Prepare homogenate

1. Homogenize 150 g diced beef muscle in a blender with 450 ml of homogenization buffer for 1 to 2 min at high speed.
2. Divide homogenate equally between tubes and centrifuge 10 min at 3000 × *g*, 4°C.
3. Pool supernatants, discard precipitate, and adjust pH of resulting supernatant to 8.0 using sodium hydroxide.
4. Filter supernatant through two layers of cheesecloth to remove lipid and connective tissue particles.

Precipitate myoglobin

5. Bring filtrate to 70% ammonium sulfate saturation (472 g ammonium sulfate/liter filtrate), adjust pH to 8.0 using sodium hydroxide, and stir for 1 hr.
6. Divide homogenate equally between tubes and centrifuge 20 min at 18,000 × *g*, 4°C to remove precipitated proteins.
7. Pool supernatants and discard precipitate.
8. Bring supernatant from 70% to 100% ammonium sulfate saturation (by adding an additional 228 g ammonium sulfate/liter supernatant), adjust pH to 8.0 using sodium hydroxide, and stir for 1 hr.

9. Divide homogenate equally between tubes and centrifuge solution 1 hr at 20,000 × *g*, 4°C. Discard supernatants.

Dialyze and purify myoglobin

10. Transfer precipitated myoglobin to dialysis tubing and dialyze against dialysis buffer (1 vol protein:10 vol buffer) for 24 hr at 4°C, changing buffer every 8 hr.
11. Equilibrate a Sephacryl S-200 HR chromatography column with chromatography elution buffer (3 column volumes) using a peristaltic pump.
12. Apply dialysate to column and resolve myoglobin extract with chromatography elution buffer at a flow rate of 60 ml/hr.

    Hemoglobin will elute first as a pale red/brown band. Myoglobin will follow as a readily visualized dark red band.

13. Collect myoglobin-containing fractions.

Concentrate myoglobin

14. Pool all myoglobin-containing fractions and bring solution to 100% ammonium sulfate saturation (761 g ammonium sulfate/liter solution), adjust pH to 8.0, and stir solution for 1 hr.
15. Divide solution equally between tubes and centrifuge 1 hr at 20,000 × *g*, 4°C. Discard supernatants and dialyze myoglobin as described in step 10.

    Alternatively, the myoglobin may be concentrated using ultrafiltration as described by Trout and Gutzke (1996).

    Native-PAGE (UNIT B3.1) can be used to assess the purity of the myoglobin extract, which should produce a single protein band with a molecular weight of 17.8 kDa.

16. Measure protein concentration of myoglobin solution (see Basic Protocol 1, step 9) and freeze in aliquots at −80°C.

PREPARATION OF OXYMYOGLOBIN BY REDUCTION OF METMYOGLOBIN

BASIC PROTOCOL 4

The basis for myoglobin oxidation in meat has often been studied using in vitro models. Commercially available myoglobin often exists in ≥95% of the ferric metmyoglobin form. Because many experiments are concerned with oxidation of ferrous oxymyoglobin, it is necessary to chemically reduce myoglobin as purchased. Additionally, this approach can be used to reduce myoglobin purified from muscle of meat-producing animals (see Basic Protocol 3), if necessary. The following procedure, adapted from Brown and Mebine (1969), involves chemical reduction of metmyoglobin with hydrosulfite. Unreacted hydrosulfite is subsequently removed via chromatography.

Materials

Myoglobin stock solution of isolated (see Basic Protocol 3) commercial myoglobin (e.g., Sigma)
Sodium hydrosulfite (sodium dithionite)
Sephadex G-25 desalting column

NOTE: It is extremely critical to perform all steps at 4°C to minimize oxidation of ferrous deoxy- or oxymyoglobin to metmyoglobin, especially when using a buffer at pH 5.6, the typical post-mortem skeletal muscle pH.

1. Treat a myoglobin stock solution (on ice) with sodium hydrosulfite at a rate of 0.1 mg sodium hydrosulfite to 1 mg myoglobin. Vortex 10 sec to reduce metmyoglobin to deoxy- and oxymyoglobin.

   Although the final concentration of myoglobin desired in the experiment must be considered, a concentrated myoglobin stock solution (~40 mM) is easily prepared and reduced. Additionally, 0.15 mM myoglobin is routinely used in the authors' experiments, as this represents an average concentration for myoglobin in different bovine skeletal muscles (Rickansrud and Henrickson, 1967).

   Commercial myoglobin should be made up with a buffer (e.g., phosphate or citrate buffer) that is suitable for the particular experimental conditions.

2. Bubble air through solution with a small pipet for 1 min to oxygenate myoglobin.

   A high quantity of oxymyoglobin is produced following oxygenation.

3. Using gravity flow, pass the myoglobin solution over a Sephadex G-25 desalting column to remove excess hydrosulfite.

   Alternatively, excess hydrosulfite may be removed from the myoglobin solution via dialysis against the buffer of choice (1 vol protein:10 vol buffer, 3 times, 8 hr each) or via mixed-bed ion-exchange chromatography (e.g., AG501-X8, Bio-Rad) (Brown and Mebine, 1969).

4. Use myoglobin immediately as oxidation can occur rapidly.

COMMENTARY

Background Information

Myoglobin is a heme protein found in the skeletal muscle of meat-producing animals. It provides the red color associated with meat and thus affects the appearance of meat. Two other heme-containing proteins found in meat are hemoglobin and cytochromes. These are generally present at relatively low concentrations and are not considered to substantially impact the color of meat from normal animals (Warriss and Rhodes, 1977; Ledward, 1984). Several reviews have been published on the biochemistry of myoglobin and its relevance in meat (Faustman and Cassens, 1990; Renerre, 1990; Cornforth, 1994).

The presence of myoglobin in meat impacts two important aspects of meat color. First, the total amount of coloration is directly proportional to the concentration of myoglobin present. Myoglobin concentration is affected by species (Livingston and Brown, 1981), animal age (Lawrie, 1974), diet (MacDougall et al., 1973), and muscle type (Lawrie, 1974). Muscles with a high proportion of red oxidative myofibers contain more myoglobin, and as animals age, the concentration of myoglobin in muscles increases. The marketing of white, or special fed, veal is based on close monitoring of the dietary iron intake in bovine calves, and restriction of this micronutrient yields muscles with reduced pigmentation (Bremner et al., 1976).

Myoglobin affects meat color stability as well as intensity. The color perceived by consumers on the surface of fresh meat is determined by the relative concentrations of the three redox forms of myoglobin. Deoxymyoglobin is a purplish pigment in which the heme iron is in a ferrous (Fe^{2+}) state with nothing bound at the sixth coordination site. Oxymyoglobin results when deoxymyoglobin is exposed to air. Oxygen binds to the sixth site of ferrous heme and produces cherry-red oxymyoglobin. Either of these ferrous myoglobins can autoxidize to brownish red metmyoglobin. Metmyoglobin contains heme iron in the ferric (Fe^{3+}) state with water bound at the sixth site. The spectra for each of the three forms of myoglobin are presented in Figure F3.3.1. It is important to note the isobestic point at 525 nm at which the spectra from all three myoglobin forms intersect. The estimation of myoglobin concentration in muscle extract is most easily accomplished at this wavelength, where a single extinction coefficient can be applied. Extinction coefficients have been determined for a number of wavelengths, and myoglobin forms and an extensive list of coefficients can be found in Bowen (1949). The wavelength at which metmyoglobin shows a strong absorbance relative to the ferrous myo-

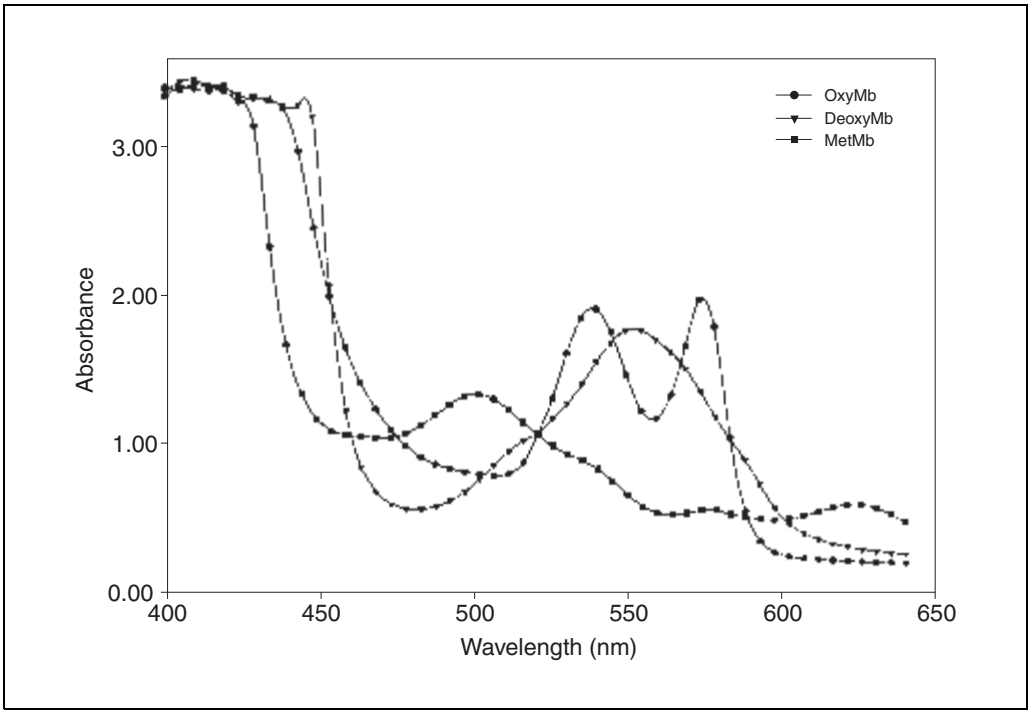

Figure F3.3.1 Absorbance spectra for deoxy-, oxy-, and metmyoglobin.

globins is 630 nm, while the wavelength at which the maximum difference in absorbance between metmyoglobin and oxymyoglobin occurs is 580 nm.

As a heme-containing protein, myoglobin shows an exceedingly intense spectral band, often called the Soret band (or *B* band), between 380 and 420 nm (Fig. F3.3.1; Gouterman, 1978). The Soret band, named after its discoverer, is attributed to the unique absorbance of the porphyrin ring, a cyclic compound formed by the linkage of four pyrrole rings through methenyl bridges (Martin, 1981).

Many factors (e.g., temperature, pH, water activity, packaging) affect the prevalence of the three myoglobin forms in meat (Faustman and Cassens, 1990) and their effects can be measured by following oxymyoglobin oxidation to metmyoglobin. For the purposes of this unit, the authors wish to emphasize the importance of both temperature and pH. Myoglobin oxidation is affected substantially by temperature with autoxidation rates higher at higher temperatures. Although myoglobin in meat is precipitated at 60°C and above (Ledward, 1971), marked denaturation of myoglobin in pure solution occurs at 65°C followed by precipitation at 70.1°C (Kristensen and Andersen, 1997). The difference in denaturation temperature can be attributed to the fact that, in meat, myoglobin coprecipitates with various other meat proteins. The pH of a myoglobin solution is also critical, and ferrous myoglobins readily oxidize to metmyoglobin as pH is decreased from physiological to postmortem meat values. In order to minimize oxidation of deoxy- or oxymyoglobin to metmyoglobin, it is critical to keep solutions cold (0° to 5°C) at all times. In addition, a high pH (8.0 to 8.5) will discourage myoglobin autoxidation; however, meat-related research often requires an experimental pH of 5.6, the typical post-mortem skeletal muscle pH.

Critical Parameters

There are several important points that need to be stressed. First, the estimation of discoloration in meat by measurement of percent metmyoglobin is useful in a relative sense only. Proper control samples (i.e., nontreated samples subjected to identical conditions, dilutions, and analyses, as treated samples) must be in place, and color measurement must occur in the same manner with both control and treated samples. Additionally, comparisons of values for percent metmyoglobin between different laboratories are only valid when investigators use the same measurement procedure. The use of three different formulas (i.e., from three different procedures) will yield different values for percent metmyoglobin. Again, it is the relative differences between treatments, or the changes over time, that become important, and

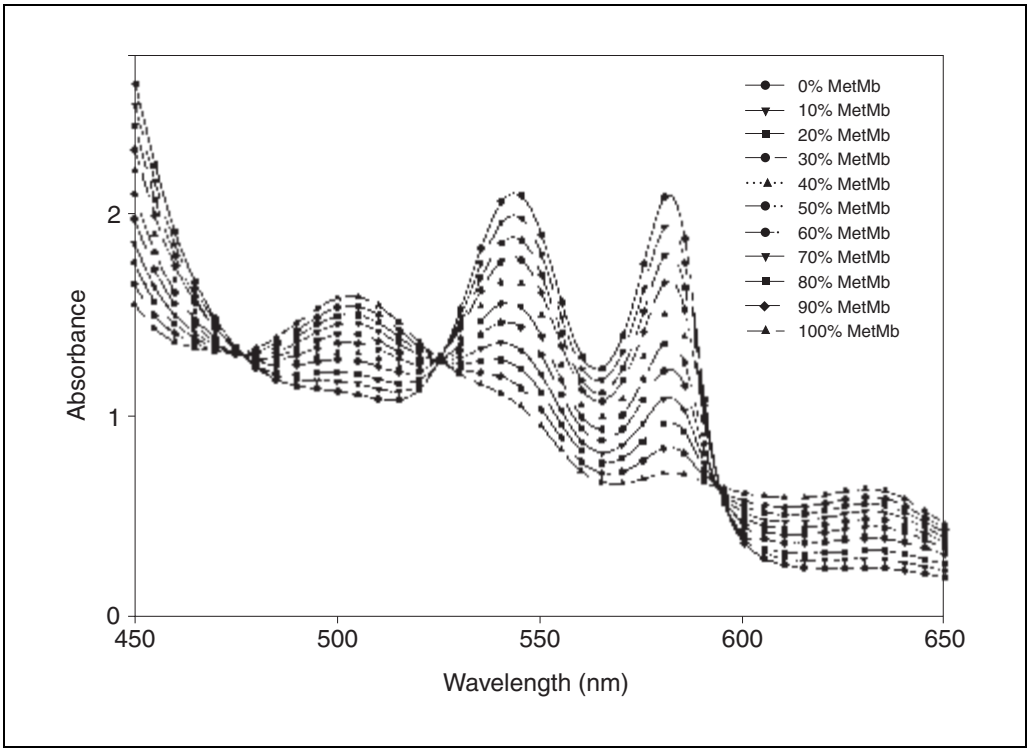

Figure F3.3.2 Absorbance spectra for myoglobin solutions containing different proportions of oxy- and metmyoglobin.

not the absolute values of metmyoglobin calculated.

As noted above, myoglobin oxidation is affected significantly by pH and temperature. At the pH of normal meat (5.6), myoglobin oxidation is more rapid than at higher pH values. It is recommended that extraction procedures utilize refrigerated conditions where possible for homogenization and filtration. The use of buffers at pH values greater than 5.6 (e.g., pH 6.8; Warriss, 1979) will also minimize any further oxidation during sample preparation. Finally, spectral analyses should be performed immediately following filtration.

Turbidity can also be problematic for some ground meat samples, as noted above. In order to avoid light scattering and erroneous results, which will occur as a result of turbidity, it is essential that proper filtration be used. In general, filtration through Whatman no. 1 filter paper is sufficient; however, when additional filtration is required, the authors have found the use of syringe-mounted filter units with 0.40-µm filters to work well.

Anticipated Results

The yield of purified myoglobin obtained with Basic Protocol 3 can vary greatly depending on the source of beef muscle used. As meat is displayed under retail display and/or storage conditions, or as myoglobin solutions are permitted to incubate, oxymyoglobin will oxidize to metmyoglobin. As such, results will be reported as metmyoglobin accumulation or oxymyoglobin loss. The units should be expressed as a percentage of the total myoglobin present rather than an absolute concentration for reasons already noted. In general, most investigators use metmyoglobin formation as a measure of meat discoloration when reporting study results. A spectrum for myoglobin containing different proportions of oxymyoglobin and metmyoglobin is presented in Figure F3.3.2. Nearly 100% oxymyoglobin is obtained if Basic Protocol 4 is followed correctly.

The use of colorimetry for evaluating discoloration in fresh meat is well established and has been reviewed elsewhere (Francis and Clydesdale, 1975; Hunt, 1980). In general, a^* values are most commonly reported, as they indicate the degree of redness in meat. Several colorimetric parameters correlate very well with sensory assessment of meat discoloration and hue angle, as shown in Figure F3.3.3.

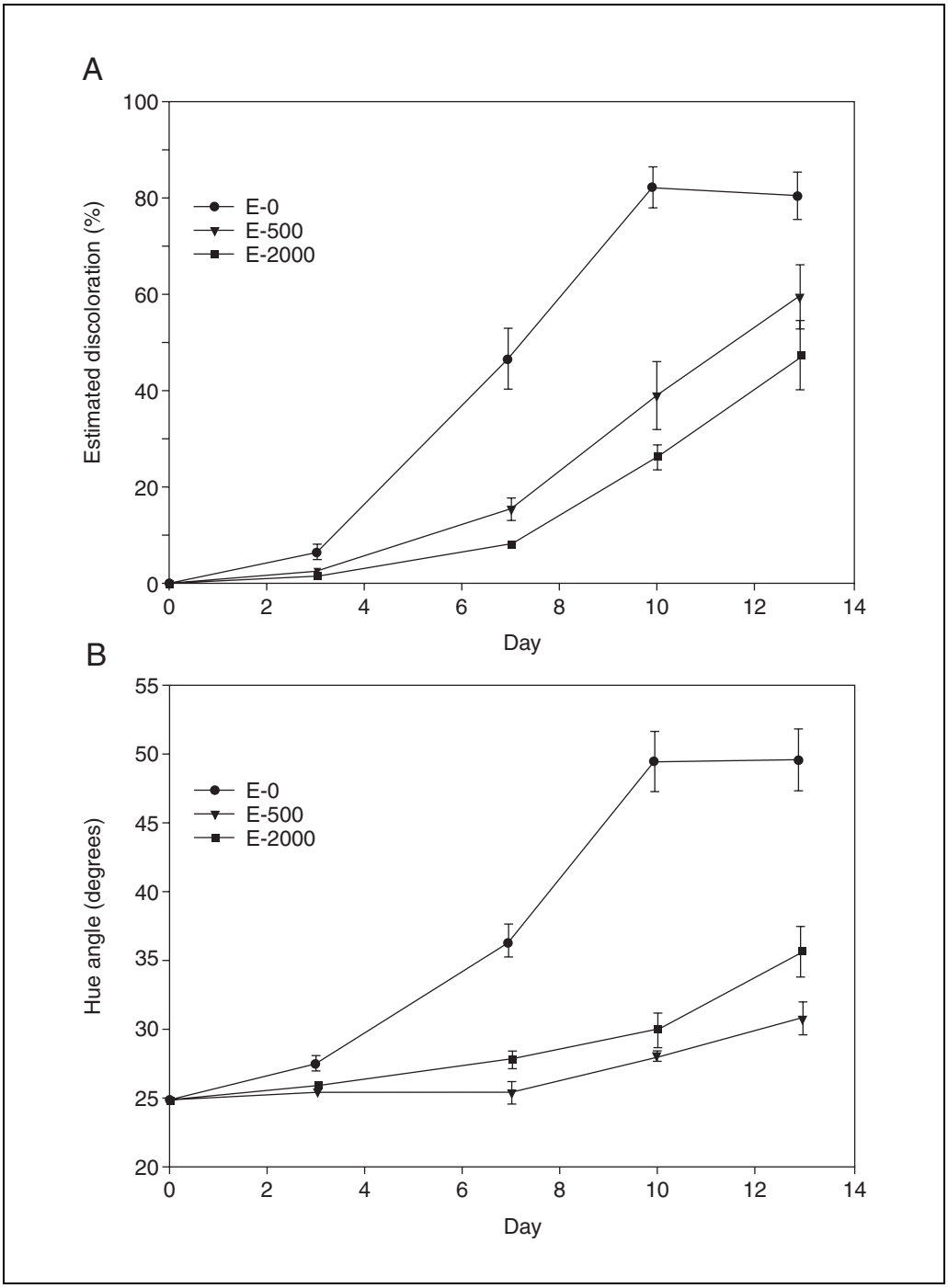

Figure F3.3.3 Comparison of subjective sensory assessment (**A**; percent discoloration) and objective colorimetric evaluation (**B**; hue angle). Beef was obtained from cattle supplemented with 0 (E-0), 500 (E-500) or 2000 (E-2000) IU α-tocopherol acetate per head per day. The α-tocopherol demonstrated a color preservation effect. Hue angle was calculated as $[\tan^{-1}(b^*/a^*)]^*(360°/2\pi)$. Standard error bars are indicated. Adapted from Chan et al. (1995), with permission from the Institute of Food Technologists.

Time Considerations

Meat discoloration studies typically involve a maximum of 5 days, with discoloration analysis being performed every day or on alternate days. The actual experimental time involved in the objective assessment of discoloration is not extensive and depends on the number of samples being analyzed. Colorimetric measurements with hand-held colorimeters are very rapid (three measurements per meat surface in <1 min). Spectral scans of meat surfaces require 1 to 2 min. Extraction and analysis of ground meat products has the added step of homogenization and filtration prior to spectrophotometry, but relative to many laboratory procedures, this is relatively quick. Isolation and purification of preparative amounts of myoglobin requires only 2 to 3 days once appropriate preparations are made. Finally, metmyoglobin can be reduced to oxymyoglobin in 15 to 20 min.

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An overview of instrumental analyses used to measure meat color and their relation to human visual appraisal.

Krzywicki, 1979. See above.

Provides equations used with reflectance spectrophotometry to calculate the relative proportions of myoglobin, oxymyoglobin, and metmyoglobin on beef surfaces.

Krzywicki, 1982. See above.

Describes a method to calculate the relative concentration of myoglobin, oxymyoglobin, and metmyoglobin present in a meat extract.

Stewart et al., 1965. See above.

The procedure upon which Basic Protocol 2 is based; the researchers utilize K/S ratios to estimate the relative presence of metmyoglobin on meat surfaces.

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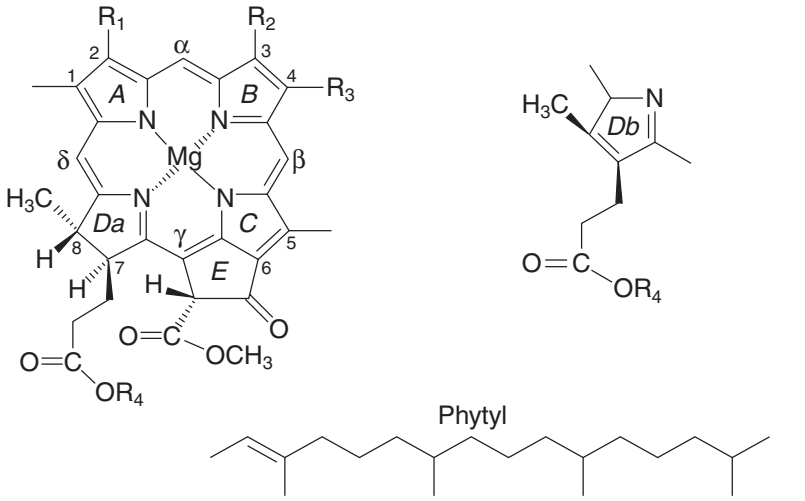
Overview of Chlorophylls in Foods

BACKGROUND AND DIVERSITY

Chlorophylls are bright green natural pigments found exclusively in photosynthetic plants and select bacteria. These pigments are based on a tetrapyrrole macrocycle linked by methene bridges, a structure which is known as a porphyrin. This basic structure maintains a high degree of unsaturation, providing an extended conjugated double-bond system that has a high metal-binding capacity (Dailey, 1990). Chlorophyll's porphyrin structure is expanded by addition of a fifth isocyclic ring (ring E) joining at position 6 and γ (Figure F4.1.1). The main modifications of the basic porphyrin structure include substitutions of methyl (CH_3) groups at positions 1, 5, and 8; a vinyl (CH_2=CH_2) at position 2; a propionic acid group esterified to a diterpene alcohol, phytol; and a centrally bound magnesium atom (Gross, 1991). From this basic structure, five classes of chlorophylls exist naturally in plants and photosynthetic organisms—*a*, *b*, *c*, *d*, and *e*—with the latter being only a minor derivative. Chlorophylls *a* and *b* predominate naturally in all higher plants, while chlorophyll *c*, *d*, and *e* derivatives are found throughout various photosynthetic algal and diatomic species including brown, red, and yellow-green algae. Additionally, four classes of bacteriochlorophylls have been isolated in photosynthetic bacteria, with bacteriochlorophyll *a* and *b* predominating in purple bacteria while *c* and *d* are found in green and purple sulfur bacteria (Sheer, 1991; Hendry, 2000).

Chlorophylls are widely distributed among green fruits and vegetables. Generally chlorophyll *a* predominates over chlorophyll *b* by a 3-to-1 margin. While native chlorophylls function mainly as primary photosynthetic pigments, their presence is considered crucial to final food product acceptance, as the green color they impart is often associated with fresh vegetable quality. Specific distribution and content of the pigments in fruits and vegetables



Pigment	Mol. Wt.	R_1	R_2	R_3	R_4	Ring D
Chlorophyll *a*	892	C_2H_3	CH_3	C_2H_5	Phytyl	a
Chlorophyll *b*	906	C_2H_3	CHO	C_2H_5	Phytyl	a
Chlorophyll *c1*	610	C_2H_3	CH_3	C_2H_5	H	b
c2	608	C_2H_3	CH_3	C_2H_3	H	b
c3	656	C_2H_3	$COOCH_3$	C_2H_3	H	b
Chlorophyll *d*	894	CHO	CH_3	C_2H_5	Phytyl	a

Figure F4.1.1 Structural differences between major classes of natural chlorophylls in higher plants and algae. Designation of pyrrole rings A-D and methene bridges α-δ is based on the nomenclature outlined by Fisher and Orth (1937).

Contributed by Mario G. Ferruzzi and Steven J. Schwartz

Current Protocols in Food Analytical Chemistry (2001) F4.1.1-F4.1.9

Table F4.1.1 Approximate Chlorophyll Content of Various Green Vegetables[a]

Tissue	Chlorophyll content (μg Chl/g vegetable tissue)		
	a	*b*	Total
Asparagus[b,c]			
Fresh tissue	139	74	180-300
Canned–Dry tissue	180 (Phe *a*) 110 (Pyro a)	51 (Phe a) 30 (pyro *b*)	
Beans[b,c]			
Fresh tissue	54	17	
Dry tissue		230-870	
Canned–Dry tissue	340 (Phe *a*) 260 (Pyro *a*)	180 (Phe *b*) 95 (Pyro *b*)	
Beet Leaf[d]			
Fresh tissue		1160	
Dry tissue		13100	
Broccoli[b,e]			
Floral–Fresh tissue		322	
	106	33	160
	110	43	
Stalk–Fresh tissue			
	36	2.0	
Celery[b]			
Leaves–Fresh tissue	1143	225	
Stalks–Fresh tissue	29	7	
Chinese mustard[d]			
Fresh tissue	210	28	
Collards[b]			
Fresh tissue	1009	216	
Cucumber[b]			
Fresh tissue	64	24	
Fenugreek Leaf[e]			
Fresh tissue		2010	
Dry tissue		15700	
Kale[b]			
Fresh tissue	1370	464	1870
Lettuce[b]			
Fresh tissue	334	62	
Mango Leaf[f]			
Fresh tissue:			
Amrapalli		2220	
Malaviyabhog		1880	
Langra		2660	
Dashehari		2630	
Okra[b]			
Fresh tissue	160	132	

continued

Table F4.1.1 Approximate Chlorophyll Content of Various Green Vegetables[a], *continued*

Tissue	Chlorophyll content (μg Chl/g vegetable tissue)		
	a	*b*	Total
Olive[g]			
Fresh tissue:			
Nevadillo	5480	120	5600
Hojiblanca	11610	180	11790
Marteña	6200	320	6520
Pararero	6060	120	6180
Picual 1	22320 (620 as Chl *a*)	2040 (1200 as Chl *b*)	24360
Peas[a,b,d]			
Fresh tissue	106	12	
Canned–Dry tissue	34 (Phe *a*) 33 (Pyro *a*)	13 (Phe *b*) 12 (Pyro *b*)	
Spinach[a,b]			
Fresh tissue	1380	440	1576
Dry tissue	6980	2490	
Canned–Dry tissue	830 (Phe *a*) 4000 (Pyro *a*)		

[a]Abbreviations: Chl, chlorophyll; Phe, pheophytin; Pyro, pyropheophytin.
[b]Gross (1991).
[c]von Elbe and Schwartz (1996).
[d]Negi and Roy (2000).
[e]Murcia et al. (2000).
[f]Pandey and Tyagi (1999).
[g]Gandul-Rojas and Minguez-Mosquera (1996).

is dependent on a number of factors including type of vegetable, stage of maturity, growing conditions, and commercial food processing (Gross, 1991). A sampling of chlorophyll content for a variety of fruits and vegetables is shown in Table F4.1.1. These values should be utilized only as a general guide, and not as an absolute indication of chlorophyll content in these green vegetable products, due to the wide biological variability and differences among varieties.

Chlorophyll content of food systems is normally expressed based on a reference system (e.g., wet or dry weight basis). While wet weight chlorophyll content is often reported, it should be considered in the context of the preparation's total moisture, as water content may potentially vary between varieties and preparations. Therefore, these values should be accompanied by moisture content of the tissue in order to account for this innate variability. Alternatively, chlorophyll content expressed on a dry weight basis can easily be compared across varieties and preparations. While dry weight content is preferred, it requires a drying step (preferably freeze drying) prior to analysis, or adjustment of the wet weight values on the basis of moisture content determinations.

CHLOROPHYLL CHEMISTRY DURING FOOD PROCESSING

The orderly and programmed natural biochemical process of chlorophyll decomposition known as senescence catabolizes nearly one billion pounds of photosynthetic pigment annually (Heaton and Marangoni, 1996). This phenomenon is most evident in the fall as leaves loose their bright green colors, and has been extensively researched, but full comprehension of the complete biochemical pathways remains incomplete. Senescing tissue excluded, chlorophylls associated with the plant structure have been found to maintain excellent stability considering the harsh photodegradative environment (Hendry, 2000). The same cannot be extended to isolated chlorophylls or those subjected to food processing and preparation, including thermal treatment and acidification (Schwartz and Lorenzo, 1990).

Degradation of chlorophylls during food processing of green fruits and vegetables has been thoroughly studied and is the subject of a number of reviews (Simpson, 1985; Schwartz

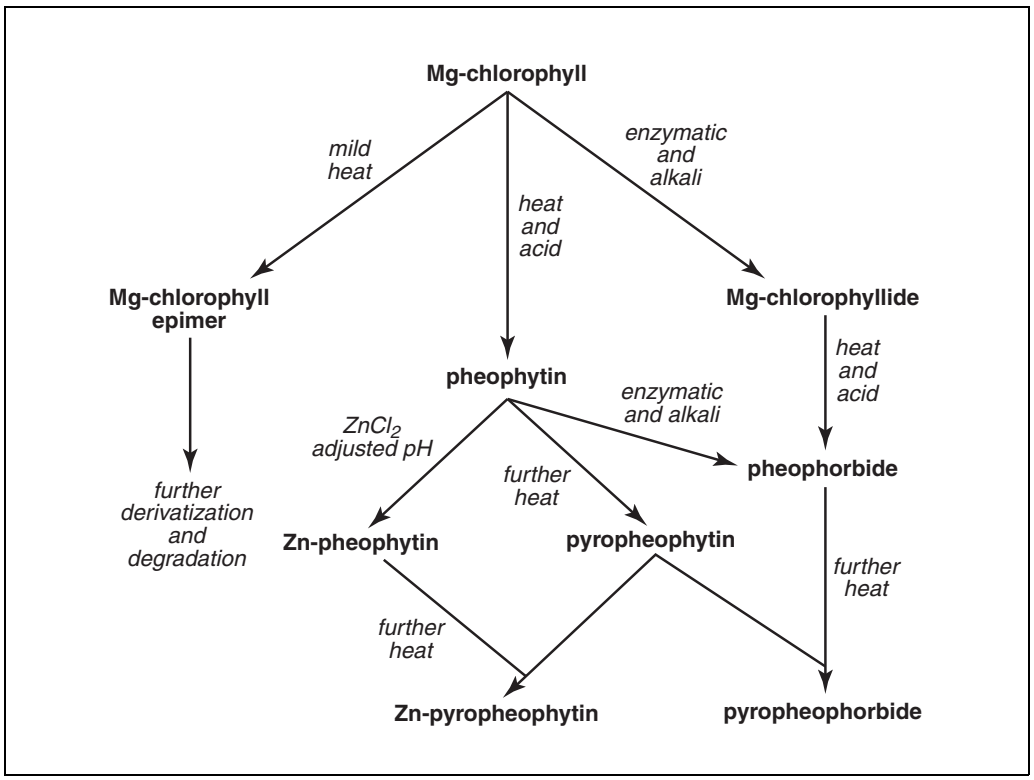
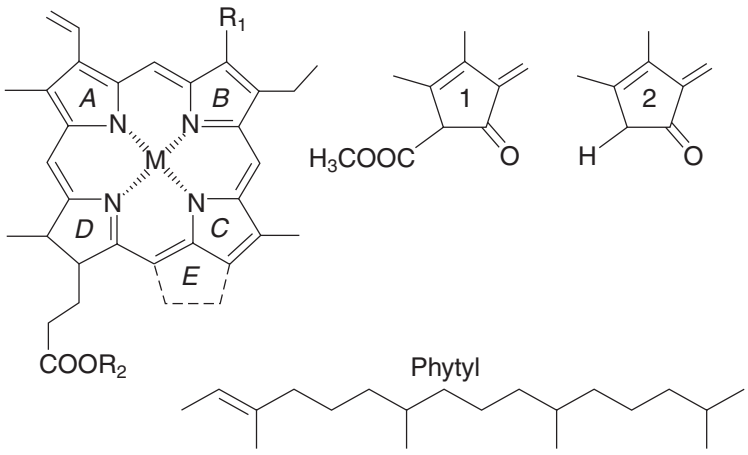


Figure F4.1.2 Major chlorophyll degradation and derivatization reactions occurring during food processing operations. Structures of major derivatives of chlorophyll *a* and *b* are depicted in Figure F4.1.3.

and Lorenzo, 1990; Heaton and Marangoni, 1996). A brief summary of the main chlorophyll reactions encountered through food processing is depicted in Figure F4.1.2, with some of the corresponding structures shown in Figure F4.1.3. Chlorophylls are extremely sensitive to physical and chemical changes encountered through food processing. These changes contribute to the perceivable discoloration of vegetable tissue from green to olive brown that is encountered during thermal processing and/or acidification. This color loss is predominantly a result of replacement of the centrally chelated magnesium atom by two atoms of hydrogen, producing metal-free pheophytin derivatives (Schwartz and Lorenzo, 1990). More severe heat treatments, as experienced in canning operations, result in the loss of C_{10} decarboxymethoxyl moeity–forming derivatives known as pyropheophytins (Schwartz et al., 1981; Schwartz and von Elbe, 1983). Pyrochlorophyll derivatives that have also been C_{10} decarboxymethoxylated but that retain the central magnesium atom have been isolated in both steamed and microwave-processed spinach leaves, but these remain less common (Teng and Chen, 1999).

Milder processing conditions also result in alteration of major chlorophyll derivatives. Formation of C_{10} epimers of the native chlorophyll structure is readily encountered during freezing, drying, and mild heating such as blanching (Katz et al., 1968; Schwartz and Lorenzo, 1990). While other conversions affect final product color, epimer formation has no detrimental effect, as the spectral properties of the epimers remain identical to those of their parent chlorophyll molecule (Sheer, 1991). Enzymatic removal of the esterified phytol by chlorophyllase results in the formation of water-soluble chlorophyllide derivatives (Schwartz and Lorenzo, 1990). These derivatives are often encountered when mild thermal treatment such as blanching is utilized, which activates chlorophyllase (Canjura et al., 1991; von Elbe and Schwartz, 1996). Further thermal processing or acidification results in formation of metal-free, water-soluble derivatives known as pheophorbides and pyropheophorbides (Schwartz and Lorenzo, 1990; Sheer, 1991).

The importance of color to final perceived quality of food products, combined with the labile nature of chlorophyll derivatives, has perpetuated numerous efforts to preserve native green vegetable appearance. Fishenbach



Derivative	Metal	R_1	R_2	Ring E
Chlorophyll *a*	Mg	CH_3	Phytyl	1
Chlorophyll *b*	Mg	CHO	Phytyl	1
Pheophytin *a*		CH_3	Phytyl	1
Pheophytin *b*		CHO	Phytyl	1
Pyropheophytin *a*		CH_3	Phytyl	2
Pyropheophytin *b*		CHO	Phytyl	2
Pheophorbide *a*		CH_3	H	2
Pheophorbide *b*		CHO	H	2
Zn-Pheophytin *a*	Zn	CH_3	Phytyl	1
Zn-Pheophytin *b*	Zn	CHO	Phytyl	1
Zn-Pheophorbide *a*	Zn	CH_3	H	2
Zn-Pheophorbide *b*	Zn	CHO	H	2
Zn-Pyropheophytin *a*	Zn	CH_3	Phytyl	2
Zn-Pyropheophytin *b*	Zn	CHO	Phytyl	2
Cu-Pheophytin *a*	Cu	CH_3	Phytyl	1
Cu-Pheophytin *b*	Cu	CHO	Phytyl	1
Cu-Pheophorbide *a*	Cu	CH_3	H	2
Cu-Pheophorbide *b*	Cu	CHO	H	2
Cu-Pyropheophytin *a*	Cu	CH_3	Phytyl	2
Cu-Pyropheophytin *b*	Cu	CHO	Phytyl	2

Figure F4.1.3 Structure of major chlorophyll derivatives prevalent in fresh and processed green vegetable tissue.

(1943) and Schanderl et al. (1965) first noticed formation of zinc and copper pheophytins and reported their increased thermal stability and color similarity with respect to natural chlorophylls. Since that time, great efforts have been dedicated to understanding the formation of these zinc and copper chlorophyll complexes for the purpose of color stabilization in processed foods. Zinc and copper pheophytin *a* and pyropheophytin *a* derivatives are rapidly formed by the addition of zinc and copper salts to commercially canned vegetables prior to thermal treatment, a process known as regreening (Schwartz and Lorenzo, 1990). Jones et al. (1977) studied the formation of copper and zinc derivatives, determining that complexation is favored at salt concentrations between one- and ten-fold the pigments' molar equivalence. The Crown Cork and Seal Company, Inc., has commercialized this canning technology for use with green beans under the trade name VERI-GREEN (Segner et al., 1984). von Elbe et al. (1986) investigated the composition of VERI-GREEN beans and determined that they consisted primarily of zinc-pheophytin *a* and zinc-pyropheophytin *a*. In subsequent studies it was determined that formation of zinc complexes was dependent on

chlorophyll species, pH, temperature, and ion concentration (LaBorde and von Elbe, 1990; Tonucci and von Elbe, 1992). Efficient formation of zinc-pheophytins and zinc-pyropheophytins in peas subjected to a continuous flow aseptic processing system raises the possibility that color preservation by metallo-chlorophyll formation may be extended beyond simple canning to more advanced processing techniques (Canjura et al., 1999).

CHLOROPHYLL ANALYSIS

The broad range of polarity offered by the diverse array of chlorophyll pigments encountered in fresh and processed fruit and vegetable products presents a true analytical challenge. Because of the labile nature of these pigments, all procedures and sample handling should be carried out in clean glassware, under subdued light, and with fresh solvents. Extraction and analysis of chlorophyll pigments from vegetable tissue is often complicated by identification of suitable solvent systems that accommodate all chlorophyll derivatives, from the polar chlorophyllides to the more apolar pheophytins and pyropheophytins (*UNIT F4.2*). Organic solvents commonly used to extract lipophilic chlorophylls, including acetone and ether, will not completely extract water-soluble derivatives such as chlorophyllides and pheophorbides, as they will remain in the aqueous phase. Recovery of these water-soluble derivatives may be efficiently accomplished by homogenization of the vegetable tissue with cold methanol followed by centrifugation for solvent clarification. Methanol extracts may be collected and vacuum-dried prior to analysis. Fresh chlorophyll extracts are extremely sensitive to degradative reactions, and should therefore ideally be analyzed immediately (*UNITS F4.3 & F4.4*). For storage, extracts may be dried, placed at subzero temperatures under a nitrogen or argon atmosphere, and protected from light (Schwartz, 1998).

Spectrophotometric Assays

Spectrophotometric assessment of chlorophyll content is based on the strong electronic absorption spectra of these pigments. Arnon (1949) developed an early method measuring 80% acetone/20% water plant extracts based on the electronic absorption spectra of chlorophylls *a* and *b*. Absorbance of the extract was measured at different wavelengths, and simultaneous equations were constructed based on extinction coefficients for each derivative's unique electronic absorption maxima. Over the last fifty years, numerous methods have appeared utilizing this approach (*UNIT F4.3*). White et al. (1965) developed equations for simultaneous determination of chlorophyll, pheophytin, and pheophorbide derivatives in plant tissues. Jones et al. (1977) describe methods for the spectrophotometric estimation of zinc pheophytins in complex mixtures with both chlorophylls and pheophytins. AOAC recognizes a spectrophotometric assay for the determination of chlorophyll *a* and *b* components in plant extracts (AOAC International, 1995).

Chromatographic Methods

Rapid and reliable chromatographic methods have been developed for analysis of complex pigment mixtures often encountered in fruit and vegetable tissues. Open-column methods based on gravity or vacuum-assisted flow have enjoyed wide application and success due to their low cost and excellent resolving power, allowing for both qualitative and quantitative separation. Tswett (1906a,b) was the first to accomplish column chromatography of chloroplast pigments using both calcium carbonate and sugar columns, providing basic separation of carotenoid and chlorophyll *a* and *b* pigments. Since that time, a number of different adsorbents have been used for the separation of chlorophyll derivatives, with sucrose in the form of powdered sugar (3% starch) having enjoyed the widest application (reviewed by Strain and Svec, 1969). Thin-layer chromatography (TLC) is a logical extension of open-column chromatography, often utilizing similar adsorbents, such as sucrose. TLC is based on utilization of glass plates coated with very thin layers of adsorbent material, which are developed by organic solvents.

Development of fast, accurate, and reproducible high-performance liquid chromatography (HPLC) methods has offset the use of traditional open-column and TLC methods in modern chlorophyll separation and analysis. A number of normal and reversed-phase methods have been developed for analysis of chlorophyll derivatives in food samples (*UNIT F4.4*), with octadecyl-bonded stationary phase (C_{18}) techniques predominating in the literature (Schwartz and Lorenzo, 1990). Inclusion of buffer salts such as ammonium acetate in the mobile phase is often useful, as this provides a proton equilibrium suitable for ionizable chlorophyllides and pheophorbides (Almela et al., 2000).

Metallo-chlorophyll derivatives such as zinc and copper derivatives present different ana-

lytical challenges as their prevalence in the food supply increases. Schwartz (1984) developed reversed-phase C_{18} methods for separation of copper and zinc pheophytins (*UNIT F4.4*). Reversed-phase C_{18} allows for effective resolution of zinc pheophytin in complex pigment mixtures including carotenoids and natural chlorophylls and pheophytins (Ferruzzi et al., 2001). Minguez-Mosquera et al. (1996) applied both TLC and reversed-phase HPLC for the determination of copper complexes of oxidized chlorophyll derivatives. Inoue et al. (1994) demonstrated the usefulness of an isocratic C_{18} method with methanol and acetic acid elution for separation of major components of water-soluble sodium copper chlorophyllin derivatives. These water-soluble metallo-derivatives have received interest for their potential health benefits, including anti-inflammatory, deodorizing, erythropoietic, antimutagenic, and antioxidant activities (Kephart, 1955; Sato et al., 1986; Harttig and Bailey, 1998).

Methods of Detection

Post-column detection of chlorophyll derivatives is often accomplished by ultraviolet and visible spectroscopic techniques, which take advantage of the strong electronic absorption spectra of these pigments (*UNITS F4.3 & F4.4*). While these methods have enjoyed wide application (Schwartz et al., 1981; Khachik et al., 1986), a major advance was made with the introduction of photodiode array (PDA) detection. Multichannel photodiode array detection allows for simultaneous monitoring of multiple wavelengths, resulting in the generation of online electronic absorption spectra of a compound as it elutes from the HPLC column. Because of the uniqueness of electronic absorption spectra of individual chlorophyll derivatives, these techniques have enjoyed extensive application for tentative identification of components from complex mixtures and extracts (*UNITS F4.3 & F4.4*).

In experiments where a higher degree of sensitivity and selectivity is required, fluorescence and mass-selective detectors have been applied. Picomole limits of detection offered by fluorescence makes it ideal for routine analysis requiring high sensitivity. Mass spectrometry has also proven to be both a sensitive and efficient way to identify numerous chlorophyll derivatives (*UNIT F4.5*). van Breemen et al. (1991) utilized both fast atom bombardment (FAB) and tandem mass spectrometry (MS/MS) for the structural characterization and mass determination of numerous derivatives including chlorophylls, chlorophyllides, pheophytins, pheophorbides, pyropheophytins, and Zn-pheophytins. Hyvärinen and Hynninen (1999) further utilized FAB-MS for identification of chlorophyll *b* allomers. HPLC with MS/MS was recently applied for identification of Cu(II)-chlorin ethyl ester in human sera (Egner et al., 2000).

FINAL CONSIDERATIONS

Analysis of chlorophylls in fruits and vegetables requires careful planning and preparation. Initial consideration must be given to sample matrix and approximate chlorophyll content. Extraction strategies will vary from tissue to tissue and with matrix status. Initial preparative steps may be required prior to extraction and analysis of samples. For example, samples of high water content such as juices may need to be dried prior to extraction. The complexity of the matrix and the nature of the analytical question will determine which techniques are most appropriate (*UNIT F4.2*). Spectrophotometric methods may be utilized in cases where quantitative determination of total chlorophyll content is desired (*UNIT F4.3*). Chromatographic techniques are appropriate when both a qualitative and quantitative chlorophyll profile is required (*UNIT F4.4*). Alteration of post-chromatographic detection allows for flexible analyses tailored to specific application end points (*UNIT F4.5*). However, extreme care must be taken at every step of the analytical process to ensure that final results are free of artifacts produced from the degradation/derivatization of these labile photosynthetic pigments.

LITERATURE CITED

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Extraction of Photosynthetic Tissues: Chlorophylls and Carotenoids

BASIC PROTOCOL

The extraction of chlorophylls and carotenoids from water-containing plant materials requires polar solvents, such as acetone, methanol, or ethanol, that can take up water. These extracts must then be transferred to a solvent such as diethyl ether in order to be stored stably. Samples with very high water content, such as juices and macerated plant material, are usually freeze-dried first, and can then be extracted directly with diethyl ether. After extraction, solutions are clarified and diluted to an appropriate volume to measure chlorophyll content by UV-VIS spectrophotometry. Absorption coefficients and equations needed for quantitative determination are given in *UNIT F4.3*.

This protocol has been developed for extraction of chlorophyll from leaf samples, but it can also be used for other plant food samples. The authors recommend, however, that the beginner initially perform the procedure using green leaf samples before extending its use to other types of samples.

NOTE: Absorption in the red and blue maxima is highest in freshly isolated chlorophyll, and then decreases with time due to formation of allomeric chlorophyll forms and possibly destruction of chlorophylls (*UNIT F4.1*), particularly in the presence of light. This also applies to green pigment extract solutions of leaves and other plant tissues. Therefore, chlorophyll determinations should be carried out in dim light immediately after preparing the pigment extract solution.

Materials

Green leaf samples or other greenish plant tissue samples
MgO or $MgCO_3$
100% or 80% (v/v) acetone or diethyl ether, spectrophotometric grade
Hydrophobic organic solvent: diethyl ether, light petrol, or hexane, spectrophotometric grade
Half-saturated NaCl solution
Anhydrous Na_2SO_4

Rim-sharpened cork driller (optional)
Mortar and pestle
Aluminum dishes
100°C drying oven
Freeze dryer
5-ml graduated centrifuge tubes
Explosion-proof tabletop centrifuge or a cooling tabletop centrifuge
UV-VIS spectrophotometer
1-cm-path-length cuvette
25- or 50-ml separatory funnel
Water bath set below 35°C (optional)

Prepare samples and determine water content

1. For each sample to be tested, prepare three to five replicates weighing 6 to 12 mg each and place in a mortar.

   Because values may vary between different parts of leaves and fruits, three to five replicates must be taken from each sample to obtain a significant and reliable mean value.

   For leaf samples, a rim-sharpened cork driller can be used to punch samples from the leaf. A punch area of 0.6 to 0.9 cm^2 will give ~6 to 12 mg fresh sample weight, equivalent to 3

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to 6 mg dry weight. For dark green leaves, a single punch for each replicate is usually sufficient to obtain good absorbance readings (0.3 to 0.85 at 662 nm). Light green leaves may require two to three punches (12 to 36 mg fresh weight) per replicate, and yellowish green leaves may require four to five punches (24 to 60 mg fresh weight) per replicate to obtain a sufficiently high absorbance reading (>0.3 at 662 nm).

For other plant food samples, appropriate sample sizes will depend on chlorophyll content and water content. Samples with low chlorophyll content may require 100 to 200 mg per replicate. For samples with low chlorophyll content and high water content (e.g., in florescences, fruit tissues, fruit juices), it is necessary to start with 2 to 3 g fresh weight sample and freeze dry it before extraction. Frozen food samples should also be freeze dried before extraction.

2. To determine the water content of the plant sample, take another four punches (24 to 48 mg sample) and weigh them. Place them in an aluminum dish, dry ~2 hr in a 100°C drying oven, and weigh them again. Subtract dry weight from original weight to obtain water content.

It is strongly recommended that the level of chlorophylls and carotenoids be determined on both a dry weight and a wet weight basis. The pigment values measured for a known leaf area can be converted to pigment values per gram dry weight sample with appropriate calculations. Dry weight and leaf area are reliable reference systems. Wet weight is less reliable since water content varies with storage.

Extract chlorophylls/carotenoids

3. Use the moisture content in step 2 to choose the appropriate extraction procedure, solvent, coefficients, and equations.

For one to five leaf punches per replicate, the water content coming from the plant tissue will account for <1% to 2% of the final 5 ml extract (step 6) and can be neglected. Perform extraction as described below and use the equations for 100% acetone (UNIT F4.3).

For 100 to 200 mg sample with very low chlorophyll, the water content in the final 5 ml acetone extract will exceed 2%. Perform extraction as described steps 4 and 5. Then, based on water content of the sample, adjust the solvent in step 6 with aqueous acetone to give a final of 5 ml of 80% aqueous acetone. Apply the equations for 80% acetone (UNIT F4.3).

For freeze-dried samples, extract the dry sample (~0.3 to 1 g dry weight starting from 2 to 3 g fresh weight) with diethyl ether and apply the equations given for diethyl ether (UNIT F4.3). Extraction of freeze-dried plant material with diethyl ether is performed by grinding in a mortar. It is also possible to use 80% acetone or 100% methanol, but diethyl ether has proved to be an excellent solvent for quantitative extraction of chlorophylls and carotenoids from freeze-dried plant material.

4. Add 100 to 200 mg MgO or $MgCO_3$ to the sample (step 1) to neutralize plant acids and prevent pheophytin *a* formation.

If high amounts of pheophytins are formed, the main absorption peaks near 660 and 662 nm would shift to other wavelengths and the green extract would change to a pale olive-green. A small amount of pheophytins (1% to 2%) does not significantly change the results.

5. Add 3 ml of 100% acetone (for ≤200 mg fresh sample) or diethyl ether (for freeze-dried samples with low chlorophyll content) and grind with a pestle.

An explosion-proof motor-driven grinder or steel or glass balls can also be used to grind the sample (see Critical Parameters).

6. Transfer the turbid pigment extract comprising chlorophylls and total carotenoids to a 5-ml graduated glass centrifuge tube. Rinse the grinding device with another 1.5 ml solvent, add to the centrifuge tube, and bring to exactly 5 ml with additional solvent.

7. Centrifuge 5 min at 300 to 500 × *g* in an explosion-proof tabletop centrifuge at room temperature.

   Slight cooling (10° to 15 °C) can be used.

Perform spectrophotometric analysis

8. Transfer an aliquot of the clear leaf extract (supernatant) with a pipet to a 1-cm-path-length cuvette and take absorbance readings against a solvent blank in a UV-VIS spectrophotometer at five wavelengths:

   750 nm (A_{750} = 0 for clear extract)
   662 nm (chlorophyll *a* maximum using 100% acetone)
   645 nm (chlorophyll *b* maximum using 100% acetone)
   520 nm (for extracts from green plant tissue, A_{520} should be <10% A_{662})
   470 nm (carotenoids).

   For other solvents, use the chlorophyll maxima, absorption coefficients, and equations found in UNIT F4.3 and in Lichtenthaler (1987).

   For green to dark green leaves, extracts made from 6 to 12 mg leaf sample give A_{662} values between 0.3 and 0.85. If $A_{662} \leq 0.3$ (e.g., with light green or yellow-green leaves), the procedure should be repeated using additional sample (see step 1).

9. Apply measured absorbance values to equations given for each solvent system in UNIT F4.3 to determine pigment content (µg/ml extract solution). Multiply by 5 ml to obtain the total amounts of chlorophyll *a*, chlorophyll *b*, and carotenoids contained in the 5 ml extract.

   This represents the µg pigment in each replicate sample.

10. Determine the mean value from each set of replicates.

Store sample

11. Prepare a fresh extract from a larger amount of tissue, or pool the remainder of the replicate pigment extracts (step 7). Transfer to a 25- or 50-ml separatory funnel. Add 3 ml hydrophobic organic solvent (diethyl ether, hexane, or light petrol) and gently shake.

    In order to obtain an extract that can, under exclusion of light and water, be stored in a refrigerator for days and weeks, the pigments should be transferred to a hydrophobic organic solvent that gives a phase separation with water, such as light petrol (a mixture of hydrocarbons, boiling point 40° to 70°C), hexane, or diethyl ether.

    A 10- to 15-ml aqueous acetone extract solution (i.e., from three replicates) or a direct extract from a larger tissue sample can be extracted with 3 ml hydrophobic solvent.

    Rigorous shaking should be avoided as it causes formation of water-lipid emulsions that can only be broken down by centrifugation.

12. Add 10 to 15 ml half-saturated NaCl solution under continued gentle shaking until the hypophase (lower phase), which contains the original organic solvent (e.g., acetone), is ~50% aqueous and the chlorophylls and carotenoids are transferred to the organic epiphase.

    The half-saturated NaCl solution prevents emulsion formation.

    Under these conditions, chlorophylls a and b, carotenoids, and xanthophyll esters are transferred to the epiphase (upper phase), as described in German in Lichtenthaler and Pfister (1978) and briefly in English in Lichtenthaler (1987).

13. Transfer hypophase to a separatory funnel and extract the last traces of chlorophylls and carotenoids using 2 ml of the hydrophobic organic solvent used in step 11.

    The transfer of photosynthetic pigments from a larger extract in an aqueous organic solvent by a small amount of a hydrophobic organic solvent concentrates the pigments.

14. Combine the epiphases from the two extractions and wash once or twice with a small amount (e.g., 1 or 2 ml) of half-saturated NaCl solution.
15. Add 100 to 200 mg anhydrous Na_2SO_4 and decant the concentrated extract into a measuring flask. Close with a glass or Teflon stopper. For larger amounts of extract, concentrate the hydrophobic epiphase to a fixed volume (e.g., 5 or 10 ml) by evaporation in a water bath set below 35°C.
16. Store extract wrapped in foil at 4°C (stable for days or weeks).

For comparison with other plant samples, take 0.05 or 0.1 ml of this concentrated pigment extract, dilute to 5 ml with 100% acetone or diethyl ether, and determine the amount of photosynthetic pigments using the absorption coefficients and equations given in UNIT F4.3.

COMMENTARY

Background Information

Before the extraction of photosynthetic pigments, the plant material should be well defined, i.e., one should think in advance about the reference system for the chlorophyll (Chl) and carotenoid concentration (e.g., mg Chl/g dry weight or mg Chl/m^2 leaf area). The reference systems frequently used are: fresh weight, dry weight, or leaf area. Dry weight is a reliable reference system. However, fresh weight generally is not a suitable reference system because it includes the water content of plant tissue (leaves, fruits), which is highly variable. For this reason, the dry weight of a parallel plant sample must be determined, so that the pigment content can be expressed on a dry weight basis, providing a much more reliable reference system than fresh weight. For leafy food samples such as lettuce and spinach, leaf area is also an acceptable reference system.

Critical Parameters

Plant material

Usually the plant material can be directly extracted without any pretreatment. In cases of extremely high water content (e.g., juices, macerated plant material), the sample should be freeze-dried before extraction; otherwise, the lipid-soluble pigments cannot be adequately extracted. In addition, the absorption maxima of pigments in organic solvents are shifted towards longer wavelengths when water is present, and the absorption coefficients are considerably changed with increasing water content of the extract (Lichtenthaler, 1987). This requires some precautions to apply the proper equations for pigment determination.

Extraction procedure

Extraction of photosynthetic pigments can be carried out using a mortar and pestle or a motor-driven grinder, or by shaking the plant material with glass or steel balls. When using a conventional grinder with an electric motor, one should be aware of the danger involved in using inflammable organic solvents (e.g., acetone). In this case, a mortar and pestle are safer, and they are also easier to clean. It is advisable to add small amounts of MgO or $MgCO_3$ to neutralize plant acids that cause the formation of pheophytin *a* from Chl *a*. It is advisable to start pigment extraction with a small volume of solvent. When the plant material is well homogenized, add more solvent to give a final defined volume of the extract solution. If too much solvent is used, the pigments will be too dilute and absorbance readings will no longer be possible to obtain. Thus, 6 to 12 mg leaf material can easily be extracted with 3 ml acetone, re-extracted with 1.5 ml acetone, and then brought to a 5-ml final volume. After centrifugation for 5 min, 3 ml of the clear extract solution are placed into a cuvette for quantitative determination in the spectrophotometer.

Water-containing plant materials need to be extracted with polar solvents such as acetone, methanol, or ethanol that can take up water. Freeze-dried plant tissues and freeze-dried juices can be directly extracted with diethyl ether, which contains traces of water and is more polar than light petrol or hexane. Pure light petrol or hexane are less suitable, because more polar pigments, such as Chl *b* or xanthophylls, are only partially extracted from freeze-dried plant samples. A few drops of acetone or ethanol added to light petrol or hexane will, however, guarantee a complete extraction. This mixture will extract Chl *a*, Chl *b*, and all carotenoids—including xanthophyll esters and secondary carotenoids that are present in many fruits and juices—from the freeze-dried plant material.

Solvent

Chlorophylls and carotenoids are generally extracted with organic solvents. One should always apply purified solvents such as spectrophotometric grades, which are commercially available. This is an essential requirement. Organic impurities in standard-grade organic solvents considerably change absorption coefficients and wavelength maxima of the pigments. Because of the relatively high water content of intact plant material, one should use a solvent that mixes with water, such as acetone, ethanol, or methanol. However, chlorophylls are unstable in these water-containing solvents. Allomeric chlorophyll types that possess different absorption characteristics can eventually form. Moreover, part of Chl *a* can be broken down to pheophytin *a* (by removal of the central Mg atom), especially in the presence of plant acids from the vacuoles of extracted plant material (*UNIT F4.1*). Pheophytin *a* exhibits quite different absorption characteristics from Chl *a*. In addition, light photochemically destructs chlorophylls and carotenoids. Avoid chloroform, which is sometimes proposed as an extraction solvent for chlorophylls, because it not only is poisonous, but also contains hydrochloric acid, which partially transforms Chl *a* into pheophytin *a*. The formation of pheophytin *b* from Chl *b* usually does not occur during extraction, as this requires stronger acids.

Diethyl ether (spectrophotometric grade) is the best epiphase solvent, as it has a higher capacity to solubilize photosynthetic pigments than light petrol or hexane. When concentrated, pigment extracts are stored in strongly hydrophobic solvents (such as light petrol or hexane) in the refrigerator, but small turbid flakes containing polar pigments form. This does not occur with diethyl ether, which can contain some water. If diethyl ether has been used in the epiphase for extracting pigments from the hypophase, the epiphase can be stored in a refrigerator at 4° to 6°C for 1 or 2 hr in the separatory funnel. Under these conditions, water dissolved in diethyl ether at room temperature separates from the diethyl ether and this water hypophase can be discarded.

Final preparation of the extract

For the quantitative determination of Chl *a* and *b*, it is not necessary to separate these two pigments prior to spectrophotometric measurement (*UNIT F4.3*), as the absorbance of the extract is measured in the red region at the wavelength positions of both Chls. From these absorbance readings, Chl *a* and *b* are calculated by a particular subtraction method. This subtraction method also applies to the determination of total carotenoids (xanthophylls and carotenes, x + c) in a total pigment extract by measuring the absorbance at 470 nm, the main absorbance region of carotenoids (*UNIT F4.3*).

However, for an exact determination of the pigment concentration, the extract must be fully transparent to avoid obtaining values that are too high for Chl *b* and total carotenoids. In most cases, the homogenized plant extract contains colorless, undissolved, very fine, solid plant material, e.g., fibers and cell wall debris. These materials make the extract turbid and scatter light, rather than absorbing light. The scattered light increases from longer to shorter wavelengths (from red region to blue). Thus, the presumed absorbance signal measured in turbid solutions by the spectrophotometer is increased differentially for individual wavelengths (see Figure F4.3.4 in *UNIT F4.3*). Hence, the concentration of pigments, calculated from these incorrect absorbance values, are too high. Also, the ratio of Chl *a* to Chl *b* shifts from 2.7 to 3.2 to incorrect lower values of 2.1 to 2.6.

In order to have a transparent extract without turbidity, the homogenized extract should be centrifuged (5 min at $300 \times g$) or filtered. When using a centrifuge, one should cool the extract in order to keep evaporation as low as possible and avoid problems with inflammable organic solvents, such as acetone or diethyl ether. (Several companies make explosion-proof tabletop centrifuges, which should be used.) Filtering through fine glass filters (e.g., G3) at a reduced pressure (e.g., a water-wheel pump) is another, more time-consuming procedure. If used, one should ensure that no pigments are left in the filter. In addition, glass filters are quickly plugged by the fine plant debris and/or MgO or $MgCO_3$ powder added during extraction, requiring an intricate clean-up procedure. Paper filters are not suitable, as they retain only the larger plant particles and are permeable to finely ground plant debris. Special filters that retain finer plant debris and MgO or $MgCO_3$ powder take too much time to use, and the pigments may be partially oxidized or photochemically destroyed. Thus, for routine analysis, centrifugation is the method of choice. When transferring the centrifuged, clear pigment extract solution with a pipet into the spectrophotometer cuvette, great care should be taken not to disturb the sedimented debris.

It is important to note that chlorophylls are converted to pheophytins in the presence of acids (*UNIT F4.1*). Formation of a significant

amount of pheophytin due to unexpectedly high amounts of endogenous acids (which is very seldom the case) is observed as a change of the green pigment extract color to a pale olive-green. When this happens, add a drop of 25% HCl to the acetone extract, which will transform all chlorophyll *a* and *b* to pheophytin *a* and *b*. Then apply the absorption coefficients and equations given by Lichtenthaler (1987) for pheophytins. A small amount (1% to 3%) of pheophytins in the green extract solution does not significantly change the absorbance readings and can be tolerated. To avoid artifactual pheophytin formation, do not handle concentrated acids in the laboratory used for pigment extraction. Traces of gaseous acids in the air can convert chlorophylls to pheophytins before and during grinding and extraction.

Samples and extracts containing chlorophylls should be protected from light at all times. For storage and during analysis, extracts should be wrapped in aluminum foil.

Anticipated Results

Real pigment differences among leaf or other plant tissues of different light adaptation or developmental stages, and among fruit tissue of different maturation or senescence, occur on both a fresh and dry weight basis. Pigment differences showing up in just one reference system may not be real, as the reference system, such as dry weight (e.g., during fruit storage) or leaf area (e.g., shrinking during water stress or enlargement during leaf expansion) may have changed.

Time Considerations

One determination involving extraction with 100% acetone, centrifugation, and absorbance readings takes ~20 min. With experience, routine measurements of six samples can be completed in 40 to 60 min.

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Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy

The quantitative determination of chlorophyll (Chl) *a*, Chl *b*, and carotenoids in a whole-pigment extract of green plant tissue by UV-VIS spectroscopy is complicated by the choice of sample, solvent system, and spectrophotometer used. The various plant pigments absorb light in overlapping spectral regions, depending on the system selected. This unit discusses methods used to account for such overlap by applying equations for accurate quantitative determination of Chl *a*, Chl *b*, and total carotenoids in the same pigment extract of leaves or fruits. General information on the spectroscopic characteristics of Chl *a* and Chl *b*, their specific absorption coefficients, and their quantitative determination in a whole-pigment extract of green plant tissues can be found in Šesták (1971) and Lichtenthaler (1987). For Chl structures, see *UNIT F4.1*.

ABSORPTION MAXIMA

Figure F4.3.1 shows the absorption spectrum of isolated Chl *a* and Chl *b* in diethyl ether. Chl *a* and *b* absorb with narrow bands (maxima) in the blue (near 428 and 453 nm) and red (near 661 and 642 nm) spectral ranges. The isolated yellow carotenoids have a broad absorption with three maxima or shoulders in the blue spectral range between 400 and 500 nm (Fig. F4.3.2).

The absorption maxima of extracted pigments strongly depend on the type of solvent and, to some degree, on the type of spectrophotometer used. For example, with increasing polarity of the solvent, the red absorption maximum of Chl *a* shifts from 660 to 665 nm, and the blue absorption maximum from 428 to 432 nm. The same also applies to Chl *b*, which shifts from 642 to 652 nm and 452 to 469 nm (see, e.g., Fig. F4.3.3 and Table F4.3.1, and Lichtenthaler, 1987). These wavelength shifts of the absorption maxima are correlated with changes in the absorption coefficients used for the quantitative determination of Chls *a* and *b* and carotenoids. For these reasons, the absorbance readings of a pigment extract must be performed at the correct wavelength position, i.e., the maxima of pure Chl *a* and pure Chl *b* in a particular solvent. Moreover, the solvent-specific extinction coefficients have to be considered by applying the corresponding equations for calculation of the pigment content. Minor differences in the positions of the wavelength maxima also exist, depending on the spectrophotometer type used. Thus, the wavelength position can differ by 1.0 or 1.5 nm.

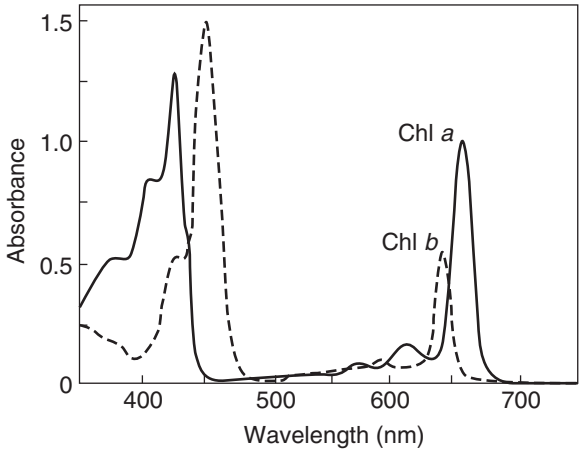

Figure F4.3.1 Absorption spectra of freshly isolated Chl *a* and Chl *b* in diethyl ether (pure solvent). The spectra were measured 40 min after extraction of pigments from leaves and 3 min after eluting the two Chls with diethyl ether from a TLC plate.

Contributed by Hartmut K. Lichtenthaler and Claus Buschmann
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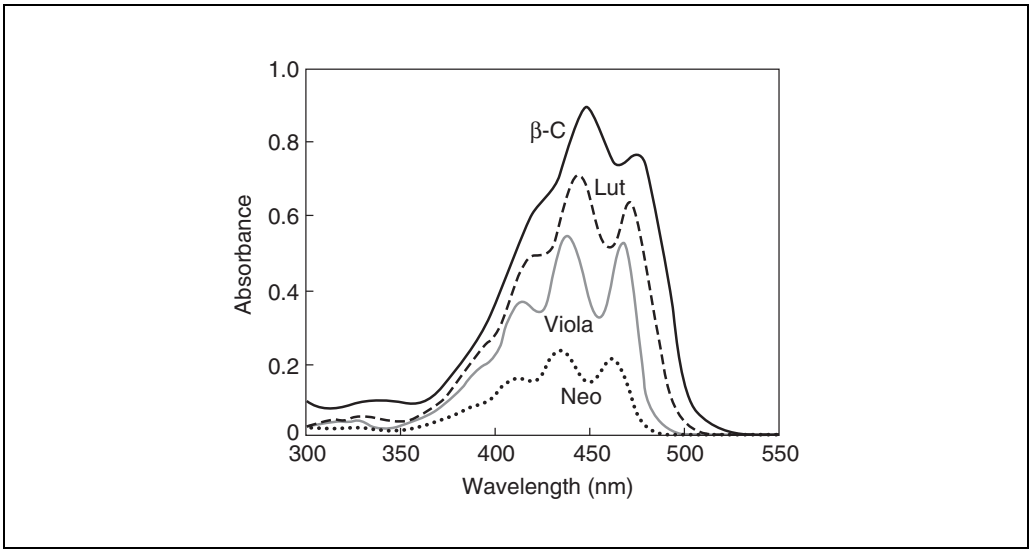

Figure F4.3.2 Absorption spectra of the major carotenoids of the photosynthetic biomembranes of green leaves of higher plants in diethyl ether (pure solvent). The carotenoids were freshly isolated from a pigment extract by TLC following Lichtenthaler and Pfister (1978) and Lichtenthaler (1987). β-C, β-carotene; Lut, lutein; Neo, neoxanthin; Viola, violaxanthin.

In order to perform spectroscopic measurements of green plant tissue extracts in the right maximum regions, one should determine the maximum red spectral position of pure Chl *a* and pure Chl *b* solutions with one's own spectrophotometer and compare them with those from the literature, given in Table F4.3.1. For a wavelength deviation of more than 1 nm, one should measure the absorbance of the pigment extract using these self-determined maxima rather than the literature values. The same equations for the particular solvent can be applied as long as wavelength positions differ by no more than 2 nm. At a deviation of >2 nm, either the spectrophotometer needs wavelength adjustment or a wrong, impure solvent has been applied. For the determination of carotenoids in the same extract solution, the wavelength position of 470 nm may be maintained, since a 1-nm shift has much less influence on the total carotenoid level than on the individual levels of Chls *a* and *b*.

ABSORPTION SPECTRA

The absorption spectrum of an extract of a green leaf containing a mixture of Chls *a* and *b* and total carotenoids (Fig. F4.3.4) is dominated by the absorption of Chl *a* at A_{428} (blue)

Table F4.3.1 Wavelength Maxima (A_{max}) and Specific Absorbance Coefficients (α)[a] of Chl *a* and *b* for Extracts in Different Organic Solvents

	Diethyl ether (water free)	Diethyl ether (pure)	Diethyl ether (water saturated)	Acetone (pure)	Acetone (with 20% water)	Ethanol (with 5% water)	Methanol (pure)
A_{max} Chl *a* [nm]	660.0	660.6	661.6	661.6	663.2	664.2	665.2
A_{max} Chl *b* [nm]	641.8	642.2	643.2	644.8	646.8	648.6	652.4
$\alpha_{(a)maxa}$	101.9	101.0	98.46	92.45	86.3	84.60	79.24
$\alpha_{(a)maxb}$	15.20	15.0	15.31	19.25	20.49	25.06	35.52
$\alpha_{(a)470}$	1.30	1.43	1.38	1.90	1.82	2.13	1.63
$\alpha_{(b)maxa}$	4.7	6.0	7.2	9.38	11.2	16.0	21.28
$\alpha_{(b)maxb}$	62.3	62.0	58.29	51.64	49.18	41.2	38.87
$\alpha_{(b)470}$	33.12	35.87	48.05	63.14	85.02	97.64	104.96
$\alpha_{(x+c)470}$	213	205	211	214	198	209	221

[a]Units of absorption coefficients are given in liter g^{-1} cm^{-1}. $\alpha_{(a)maxa}$ is the specific absorbance coefficient of Chl *a* at its red maximum; $\alpha_{(a)maxb}$ is the specific absorbance coefficient of Chl *a* at the red maximum of Chl *b*; $\alpha_{(a)470}$ is the specific absorbance coefficient of Chl *a* at 470 nm; $\alpha_{(x+c)470}$ is the specific absorbance coefficient of the sum of xanthophylls and carotenes at 470 nm.

and A_{661} (red). Chl *b* and the carotenoids absorb broadly in the blue region (400 to 500 nm).

A plant sample homogenized with an organic solvent is usually turbid and must be filtered or centrifuged to become fully transparent (see *UNIT F4.2*). Turbidity and light scattering lead to a higher absorption between 400 and 800 nm, with a slight but continuous increase towards shorter wavelengths (Fig. F4.3.5). Thus, measuring a turbid extract leads to an overestimation of the pigment levels, especially for Chl *b* and total carotenoids. Turbidity can be checked by measuring A_{750} and A_{520}. For a fully transparent leaf pigment extract, A_{750} should equal zero, since Chls *a* and *b* and carotenoids do not absorb in this region. A_{520} readings for extracts of green plant tissue should be <10% of the main Chl absorbance in the red maximum near 661 nm (diethyl ether) or 650 nm (ethanol), as shown in Figures F4.3.4 and F4.3.5.

ACCURACY OF SPECTROSCOPIC MEASUREMENTS

In order to have an exact spectroscopic measurement of absorbances, one must consider the absorbance range in which readings are made. Absorbance should be measured between 0.3 and 0.85. Leaf extracts with an absorbance <0.3 in the red region do not yield correct pigment values. There are several interfering factors, such as a base line that is not fully zeroed. Thus, values <0.3, whether read by the experimenter or given as digital values by the instrument, are not acceptable. Absorbance values >0.9 may indicate problems with the accuracy of the detector (e.g., a photomultiplier). Since the detector system examines the transmitted light of the cuvette, the absorbance is calculated from this value. When transferring the linear transmission unit to the logarithmic absorbance unit, the accuracy is exponentially reduced with rising values.

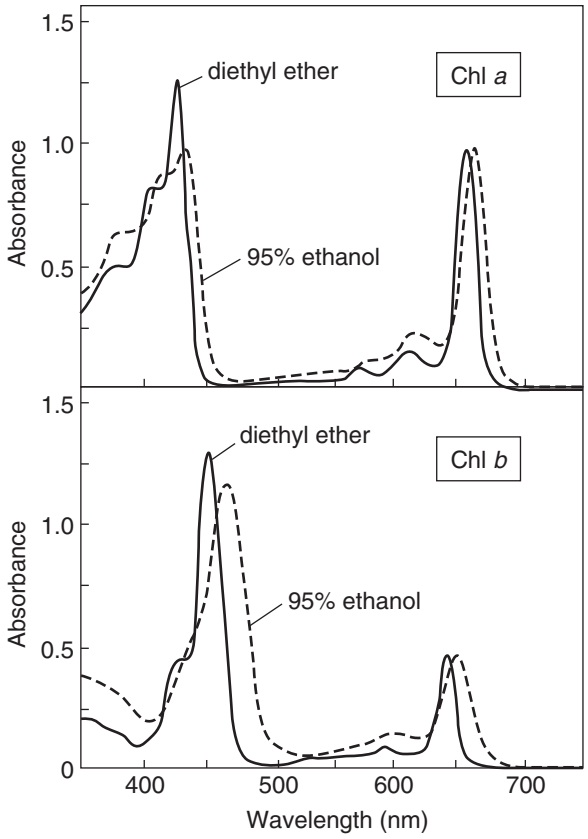

Figure F4.3.3 Differences in the absorption spectra of Chl a and Chl b in diethyl ether and 95% aqueous ethanol. For the more polar solvent (95% ethanol; broken line), the absorbance (extinction) in the blue and red absorption maxima of both Chls are decreased compared to values obtained using the less polar solvent diethyl ether (black), and the wavelength positions of the maxima are shifted to the right. For a better comparison, the absorbances in the red maxima were set at the same values.

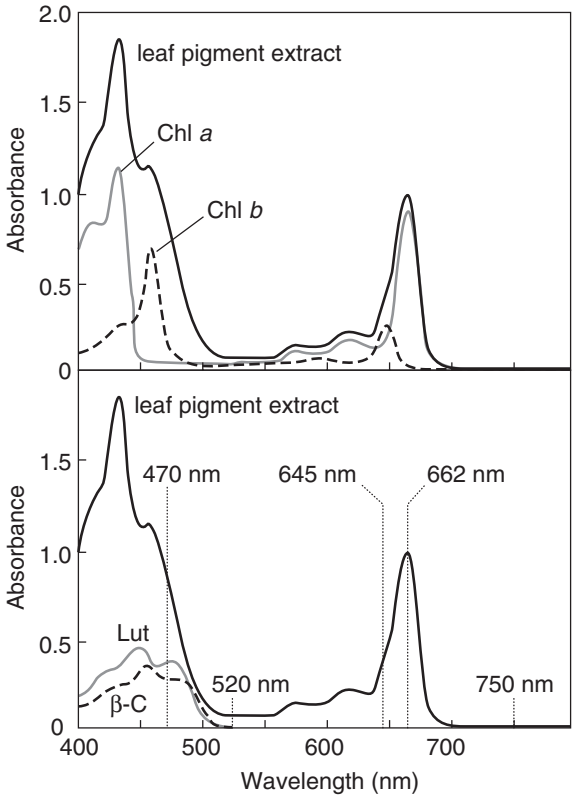

Figure F4.3.4 Absorption spectra of pigments from a green tobacco leaf extracted with 100% acetone. The leaf extract was measured directly after extracting the leaf. Chl *a*, Chl *b*, and the carotenoids β-carotene (β-C) and lutein (Lut) were measured after separation by TLC.

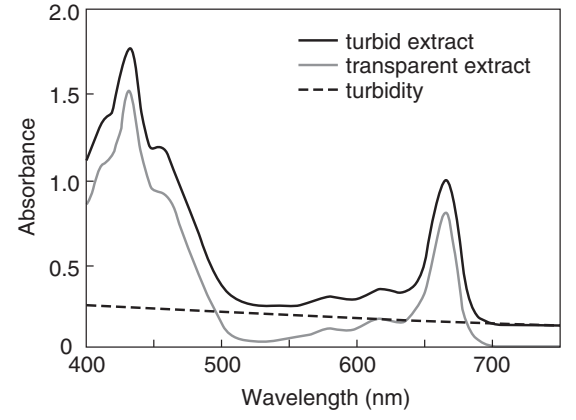

Figure F4.3.5 Absorption spectra of a leaf extract before (turbid) and after (transparent) centrifugation in 100% acetone. The difference spectrum between the two extracts represents the spectrum of turbidity.

For absorbance values <0.3, one should try to concentrate the extract (e.g., by evaporation), make a new extract using more plant material and less solvent, or extract the pigments in a separatory funnel into a small volume of a hydrophobic solvent in the epiphase. Various spectrophotometers are constructed to measure absorbance (extinction) values only up to 1.0 (i.e., a transmittance of 10%). In such cases, an absorbance >0.85 is not suitable, and the extract solution should be diluted to obtain valid Chl *b* and carotenoid values. In both cases, care must be taken to ensure that the final volume of the extract solution is carefully recorded and considered in the calculation of total Chls and carotenoids.

The extinction coefficients and the equations used and established by Arnon (1949) are not correct. They provide only a rough estimate of Chl *a* and *b* levels and yield inaccurate Chl *b* values, and, consequently, incorrect values for the Chl *a*/*b* ratio. They have been redetermined by Lichtenthaler (1987) using the extinction coefficients of Smith and Benitez (1955) for pure Chl *a* and Chl *b* in diethyl ether, which were found to be correct in the red absorption maxima at 661 and 642 nm, respectively, for purified Chls. The relative absorptions of Chl *a* and Chl *b* at other wavelengths in other organic solvents have been redetermined using modern high-resolution spectrophotometers.

To exactly determine carotenoids by measuring A_{470}, one needs to know the exact level of Chl *b*, which (in contrast to Chl *a*) also absorbs considerably at this wavelength (Fig. F4.3.1). If Chl *b* is overestimated, the level of total carotenoids becomes too low, and vice versa. With the redetermined extinction coefficients, the new equations permit the determination of total carotenoids in addition to Chl *a* and Chl *b* in the same green tissue extract solutions.

QUANTIFACTION OF PIGMENTS

The basis for spectroscopic quantification of pigments is the Lambert-Beer law, which defines the absorbance of a solution with respect to the specific light absorption characteristic of an individual dissolved compound:

$$A = \alpha c_w d \text{ or } A = \varepsilon c_m d$$

where A is absorbance (dimensionless), α is the specific absorbance coefficient in liter g^{-1} cm^{-1}, ε is the molar absorbance coefficient in liter mol^{-1} cm^{-1}, c_w is the weight concentration in g $liter^{-1}$, c_m is the molar concentration in mol $liter^{-1}$, and d is the path length of the cuvette in cm, usually 1 cm.

This original Lambert-Beer law can only be applied for one isolated pigment. Absorbance coefficients taken from the literature (Table F4.3.1) are valid only for one substance (e.g., Chl *a*) using one solvent (e.g., 100% acetone) and one wavelength (e.g., 661.6 nm). Changes in substance, solvent, or wavelength lead to changes in the absorbance coefficient.

When the concentration of Chl *a* and Chl *b* is determined from a pigment extract containing both Chls, the equation derived from the Lambert-Beer law becomes more complex. The absorbance is then expressed as the sum of the absorbances of Chl *a* and Chl *b*. Thus, the absorbance of Chl *b* contributes to the absorbance of Chl *a* at the Chl *a* maximum, and vice versa:

$$A_{\max a} = A_{(a)\max a} + A_{(b)\max a} = \left(\alpha_{(a)\max a} \times c_{ma} \times d\right) + \left(a_{(b)\max a} \times c_{mb} \times d\right)$$

$$A_{\max b} = A_{(a)\max b} + A_{(b)\max b} = \left(\alpha_{(a)\max b} \times c_{ma} \times d\right) + \left(a_{(b)\max b} \times c_{mb} \times d\right)$$

The concentrations for Chl *a* (c_a) and Chl *b* (c_b) are then given by a different equation, where the specific contribution of Chl *b* to the Chl *a* maximum and of Chl *a* to the Chl *b* maximum are subtracted. The following equations contain the denominator z, a term formed from the four extinction coefficients of Chl *a* and Chl *b*. The light path length (usually 1 cm) is omitted here:

$$c_a = \left[\frac{\alpha_{(b)\max b} \times A_{\max a}}{z}\right] - \left[\frac{\alpha_{(b)\max a} \times A_{\max b}}{z}\right]$$

$$c_b = \left[\frac{\alpha_{(a)\max a} \times A_{\max b}}{z}\right] - \left[\frac{\alpha_{(a)\max b} \times A_{\max a}}{z}\right]$$

$$z = \left(\alpha_{(a)\max a} \times \alpha_{(b)\max b}\right) - \left(\alpha_{(a)\max b} \times \alpha_{(b)\max a}\right)$$

DETERMINATION OF TOTAL CAROTENOIDS

In an extract of plant material containing carotenoids (x + c = xanthophylls and carotenes) in addition to Chls, A_{470} (the carotenoid region) is determined as the sum of specific

absorbances for Chl *a*, Chl *b*, and total carotenoids:

$$A_{470} = A_{(x+c)470} + A_{(a)470} + A_{(b)470}$$

From this follows, according to the Lambert-Beer law:

$$A_{(a)470} = \alpha_{(a)470} \times c_a \times d$$

$$A_{(b)470} = \alpha_{(b)470} \times c_b \times d$$

$$A_{(x+c)470} = \alpha_{(x+c)470} \times c_{(x+c)} \times d$$

The concentration of carotenoids $c_{(x+c)}$ is then given by the following equation, which has been reduced using d = 1 cm:

$$c_{(x+c)} = \frac{A_{(a)470} - \left(\alpha_{(a)470} \times c_a\right) - \left(\alpha_{(b)470} \times c_b\right)}{\alpha_{(x+c)470}}$$

The concentrations for Chl *a* (c_a), Chl *b* (c_b), and the sum of leaf carotenoids (c_{x+c}) can be calculated with the following equations given for different solvents, where the pigment concentrations are given in µg/ml extract solution.

Diethyl ether (pure solvent):

$$c_a\,(\mu g/ml) = 10.05\,A_{660.6} - 0.97\,A_{642.2}$$
$$c_b\,(\mu g/ml) = 16.36\,A_{642.2} - 2.43\,A_{660.6}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.43\,c_a - 35.87\,c_b)/205$$

Diethyl ether (water free):

$$c_a\,(\mu g/ml) = 9.93\,A_{660.6} - 0.75\,A_{641.8}$$
$$c_b\,(\mu g/ml) = 16.23\,A_{641.8} - 2.42\,A_{660.6}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.30\,c_a - 33.12\,c_b)/213$$

Diethyl ether (water saturated):

$$c_a\,(\mu g/ml) = 10.36\,A_{661.6} - 1.28\,A_{643.2}$$
$$c_b\,(\mu g/ml) = 17.149\,A_{643.2} - 2.72\,A_{661.6}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.38\,c_a - 48.05\,c_b)/211$$

Ethanol with 5% (v/v) water:

$$c_a\,(\mu g/ml) = 13.36\,A_{664.1} - 5.19\,A_{648.6}$$
$$c_b\,(\mu g/ml) = 27.43\,A_{648.6} - 8.12\,A_{664.1}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 2.13\,c_a - 97.64\,c_b)/209$$

Acetone (pure solvent):

$$c_a\,(\mu g/ml) = 11.24\,A_{661.6} - 2.04\,A_{644.8}$$
$$c_b\,(\mu g/ml) = 20.13\,A_{644.8} - 4.19\,A_{661.6}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.90\,c_a - 63.14\,c_b)/214$$

Acetone with 20% (v/v) water:

$$c_a\,(\mu g/ml) = 12.25\,A_{663.2} - 2.79\,A_{646.8}$$
$$c_b\,(\mu g/ml) = 21.50\,A_{646.8} - 5.10\,A_{663.2}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.82\,c_a - 85.02\,c_b)/198$$

Methanol (pure solvent):

$$c_a\,(\mu g/ml) = 16.72\,A_{665.2} - 9.16\,A_{652.4}$$
$$c_b\,(\mu g/ml) = 34.09\,A_{652.4} - 15.28\,A_{665.2}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.63\,c_a - 104.96\,c_b)/221$$

Methanol with 10% (v/v) water:

$$c_a\,(\mu g/ml) = 16.82\,A_{665.2} - 9.28\,A_{652.4}$$
$$c_b\,(\mu g/ml) = 36.92\,A_{652.4} - 16.54\,A_{665.2}$$
$$c_{(x+c)}\,(\mu g/ml) = (1000\,A_{470} - 1.91\,c_a - 95.15\,c_b)/225$$

INTERPRETATION OF CHLOROPHYLL AND CAROTENOID CONTENT

The concentration of Chl *a* and *b* in plant material can be quantified with different reference systems. Reference systems currently in use include mg Chl *a*+*b*/m^2 leaf area (or µg/cm^2 leaf area), µg Chl *a*+*b*/g dry weight, and mg Chl *a*+*b*/g fresh weight (less suitable than dry weight).

When comparing results with those of other groups or with values obtained previously, the same reference system must be applied. Changes in Chl content should be demonstrated by means of a reference that does not change, otherwise an observed variation of data may not be due to changes in Chl concentration, but instead to changes in the reference system. For instance, an increase in Chl per fresh weight (in leaves or fruits) could be solely due to a decrease in fresh weight caused by water loss. In various cases, the number of leaves, cotyledon pairs, seedlings (shoots), or fruits may be the best reference system to follow changes in pigment levels, as these numbers do not change when dry weight or leaf area vary.

The weight ratio of Chl *a* and Chl *b* (Chl *a*/*b* ratio) is an indicator of the functional pigment

Table F4.3.2 Leaves with High Versus Low Chlorophyll *a*/*b* Ratios

High *a*/*b* ratio	Low *a*/*b* ratio
Greening of etiolated leaves (4.0-10)	Fully developed green leaves (2.5-3.5)
Sun leaves (3.0-3.8)	Shade leaves (2.4-2.7)
Leaves of C_4 plants (3.0-5.0)	Leaves of C_3 plants (2.5-3.5)

equipment and light adaptation of the photosynthetic apparatus (Lichtenthaler et al., 1981). Chl *b* is found exclusively in the pigment antenna system, whereas Chl *a* is present in the reaction centers of photosystems I and II and in the pigment antenna. Whereas the light-harvesting pigment protein LHC-I of the photosynthetic pigment system PS I has an *a*/*b* ratio of ~3, that of LHC-II of PS II exhibits an *a*/*b* ratio of 1.1 to 1.3. The level of LHC-II of PS II is variable and shows a light adaptation response. Shade plants possess much higher amounts of LHC-II than sun-exposed plants and, consequently, their *a*/*b* ratios are lower than in sun-exposed plants (Lichtenthaler et al., 1982, 1984). Thus, a decrease in the Chl *a*/*b* ratio may be interpreted as an enlargement of the antenna system of PS II. Some examples for high and low Chl *a*/*b* ratios in leaves of different developmental stages and in fully differentiated leaves grown at low light or high light conditions are given in Table F4.3.2.

The weight ratio of Chls *a* and *b* to total carotenoids (*a*+*b*)/(x+c) is an indicator of the greenness of plants. The ratio (*a*+*b*)/(x+c) normally lies between 4.2 and 5 in sun leaves and sun-exposed plants, and between 5.5 and 7.0 in shade leaves and shade-exposed plants. Lower values for the ratio (*a*+*b*)/(x+c) are an indicator of senescence, stress, and damage to the plant and the photosynthetic apparatus, which is expressed by a faster breakdown of Chls than carotenoids. Leaves become more yellowish-green and exhibit values for (*a*+*b*)/(x+c) of 3.5, or even as low as 2.5 to 3.0 as senescence progresses. Also, during chromoplast development in ripening fruits or fruit scales, which turn from green to yellow or orange or red, the ratio (*a*+*b*)/(x+c) decreases continuously and reaches values below 1.0.

Sun leaves of different trees exhibit average Chl *a*+*b* levels of 400 to 700 mg/m^2 leaf area (40 to 70 µg/cm^2) and shade leaves have 380 to 570 mg/m^2 leaf area (38 to 57 µg/cm^2). As sun leaves possess thicker cell walls, a lower leaf

Table F4.3.3 Examples of Chlorophyll and Carotenoid Levels and Pigment Ratios in Green Sun and Shade Leaves[a]

Leaf type		*a* + *b* (mg/m^2)	x + c (mg/m^2)	*a* + *b* (mg/g dw)	x + c (mg/g dw)	*a*/*b*	(*a* + *b*)/(x + c)
Fagus sylvatica	Sun leaves	510.8	126.4	6.29	1.56	3.22	4.04
(beech)	Shade leaves	450.1	85.8	12.01	2.29	2.65	5.25
Carpinus betulus	Sun leaves	571.0	117.4	8.15	1.68	3.20	4.86
(hornbeam)	Shade leaves	431.1	70.8	19.05	3.13	2.45	6.09
Populus nigra (poplar)	Dark green sun leaves	724.4	161.5	8.03	1.81	3.30	4.44
	Dark green shade leaves	568.2	109.2	12.41	2.39	2.74	5.20
	Green senescent leaves	351.5	87.4	5.00	1.24	3.08	4.02
	Yellowish-green senescent leaves	140.3	79.4	1.99	1.13	3.29	1.77

[a]Pigment levels given in mg/m^2 leaf area and in mg/g dry weight (dw). Values measured are those from fully developed leaves in June, 2000. Pigment levels within one leaf usually vary by <3%, and pigment ratios vary by <1%. Abbreviations: *a* + *b*; total chlorophylls *a* and *b*; x + c, xanthophylls and carotenes (total carotenoids).

water content (50% to 65% fresh weight), and higher dry weight than shade leaves, they exhibit on a dry weight basis a considerably lower Chl and carotenoid content than shade leaves (Table F4.2.3). The latter, in turn, possess a higher water content (68% to 85% fresh weight) and, consequently, a lower dry weight than sun exposed leaves.

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Presents a table (Table 7) of chloropyll, carotenoid, and vitamin E levels (in µg/g dw) of green leaf tissue, vegetables, green and red fruits (tomato, red pepper), and nongreen plant foods (carrots, cauliflower).

Lichtenthaler, 1987. See above.

Presents redetermined absorption coefficients for chlorophylls and total carotenoids, which allows the determination of all three in the same pigment extract of leaves or fruits

Šesták, 1971. See above.

Gives basic information on the measurements of chlorophylls in various spectroscopic instruments.

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Chromatographic Separation of Chlorophylls

Numerous methods for the analysis of chlorophyll in plant tissues have been developed. Traditionally, chromatographic techniques have always been extensively utilized for analysis of these green photosynthetic pigments. While methods such as open-column and thin-layer chromatography have been extensively utilized in the past, high-performance liquid chromatography (HPLC) has dominated separation techniques over the last 20 years. The fast, accurate, and reproducible nature of these methods makes them ideal for both research and quality assurance needs. Many normal- and reversed-phase methods have been developed for analysis of chlorophylls and their derivatives. Over the last few decades, numerous procedures employing an octadecyl-bonded stationary phase (C18) have appeared in the literature. These reversed-phase (RP) methods have predominated mainly because of their ease of use, aqueous mobile phases, and the wide commercial availability of the stationary phase, making published methodology extremely transferable and reproducible.

Major method development has concentrated on the analysis of chlorophylls and their degradation products, specifically pheophytins and pyropheophytins, since they are readily formed by thermal processing of food such as spinach and green beans. The Basic Protocol described in this unit specifically targets the separation and identification of all major chlorophyll derivatives including chlorophylls *a* and *b*, pheophytins *a* and *b*, and pyropheophytins *a* and *b*. Similar methodology has been developed for the analysis of specific polar derivatives and Cu^{2+} and Zn^{2+} derivatives, and is described in the Alternate Protocol. For a general discussion and structures of chlorophylls and their degradation products, see *UNIT F4.1*.

NOTE: Chlorophylls and their derivatives are both light and temperature sensitive. All work should be carried out under subdued lighting and at a controlled ambient temperature to avoid their degradation.

STRATEGIC PLANNING

When discussing analysis by HPLC, chlorophylls may be divided into four main groups as follows.

1. Predominant natural chlorophylls *a* and *b*.
2. Nonpolar chlorophyll derivatives such as pheophytins and pyropheophytins.
3. Polar chlorophyll derivatives such as chlorophyllides and pheophorbides.
4. Major metalloporphyrin derivatives such as Cu^{2+} and Zn^{2+} pheophytins.

The protocols described in this unit focus on the separation of these four groups. While the Basic Protocol describes a method capable of separating all constituents of groups 1 and 2, it may not be perfectly suited for analysis of all polar derivatives. Therefore, it is important to have an idea in advance of which chlorophyll compounds may be in the test sample, to allow for the application of the appropriate protocols. Prior to analysis, it is important to have an estimate of the sample extract concentration to allow for optimal use of the described protocols. Such prior knowledge allows better judgment of injection and dilution volumes, which are important factors for good resolution of chlorophyll derivatives. Finally, general laboratory conditions (e.g., subdued lighting and ambient temperature) should be carefully controlled when extracting and working with chlorophylls (*UNIT F4.2*).

Contributed by Mario Ferruzzi and Steve Schwartz
Current Protocols in Food Analytical Chemistry (2001) F4.4.1-F4.4.11

BASIC PROTOCOL

C18 REVERSED-PHASE HPLC SEPARATION OF CHLOROPHYLLS *a* AND *b* AND THEIR NONPOLAR DERIVATIVES

This protocol focuses on the analysis of chlorophyll *a* and *b*, and the more nonpolar derivatives, including pheophytins and pyropheophytins. An octadecyl-bonded, reversed-phase stationary phase is used with a methanol/water mixture and ethyl acetate mobile phases in a gradient elution to provide rapid and complete separation of the major chlorophyll derivatives in 25 to 30 min. This is coupled with traditional UV/visible spectrophotometric detection at 654 nm to selectively screen these photosynthetic pigments in food and plant tissues.

Materials

HPLC-grade solvents:
- Methanol
- H_2O
- Ethyl acetate
- Acetone

Sample

Appropriate chlorophyll standards (Sigma-Aldrich; also see *UNIT F4.2*)

High-performance liquid chromatograph (HPLC) including:
- Appropriate gradient-capable solvent delivery system
- Sample injection valve
- Variable wavelength UV/VIS spectrophotometer suitable for detection at 654 nm
- Chromatographic data collection system
- Precolumn solvent filter
- Appropriate HPLC guard column and analytical column packed with octadecyl-bonded (C18) stationary phase (e.g., μBondapak C18, Waters; 201TP54, Vydac)

5-μm syringe filters

Disposable 5-ml syringes

Additional reagents and equipment for sample preparation (*UNIT F4.2*) and HPLC (e.g., Coligan et al., 2000)

Set up HPLC system

1. Set up an HPLC according to the manufacturer's instructions.

   Coligan et al. (2000), provides much information about general HPLC procedures.

2. Purge system fully of air and then install a guard column and an analytical column in-line.
3. Place a precolumn solvent filter between the guard column and analytical column.

   Use of a guard column is considered standard for HPLC as it greatly extends analytical column life.

4. Equilibrate analytical column ≥15 min with appropriate initial gradient conditions as described in Table F4.4.1 or Table F4.4.2.

   All solvents should be fully degassed by vacuum, helium sparging, or sonication prior to use.

   Ternary/quaternary versus binary solvent capabilities are instrument dependent.

5. Monitor baseline signal at 654 nm during equilibration to ensure that it is stable.

   For highly sensitive analyses, allow extra time (e.g., up to 1 hr) for equilibration to ensure an extremely stable baseline.

6. Check data collection system to ensure that it is operational.

Prepare sample

7. Extract chlorophylls from a sample as described in *UNIT F4.2*.
8. Dissolve dry extracts in 100% acetone.

   The volume of acetone is dependent on the original concentration of chlorophyll in the sample, the volume of the dried extract, and the desired concentration range for the analysis. Highly concentrated samples such as spinach may need to be diluted to ensure adherence to the linear range as prescribed by the response curves for each specific chlorophyll.

9. Pass extract through a 5-µm filter using a disposable 5-ml syringe.

Perform HPLC analysis

10. Load appropriate volume of filtered sample onto injector valve according to manufacturer's instructions.

    Injection volume will vary according to sample. For typical HPLC systems, injection volumes range between 1.0 and 100 µl. It is important that the analytical column not be overloaded. Therefore, when exploring conditions for a new sample, always start with lower injection volumes and adjust to high volumes as needed. As a rule of thumb for most analyses, injection volumes between 25 and 50 µl work best.

11. Program solvent gradient conditions as described in Table F4.4.1 or Table F4.4.2.

    As indicated in Tables F4.4.1 and F4.4.2, all gradients in this unit are simple linear gradients unless noted otherwise. This is to allow for maximum application of the described methodology to different HPLC systems.

12. Initiate chromatographic run as described by manufacturer.
13. Collect chromatographic data from specified collection system as described by manufacturer.

    Identification of chlorophylls and their derivatives can be made more efficient by use of a photodiode array detector, which allows collection of on-line spectra. However, this

Table F4.4.1 Linear Gradient Conditions for Separation of Chlorophylls and Their Nonpolar Derivatives Using a Ternary/Quaternary Solvent Delivery System

Time (min)	Flow rate (ml/min)	% Methanol	% Water	% Ethyl acetate
0.0	1.0	75.0	25.0	0.0
10.0	1.0	37.5	12.5	50.0
25.0	1.0	37.5	12.5	50.0
30.0	1.0	75.0	25.0	0.0

Table F4.4.2 Linear Gradient Conditions for Separation of Chlorophylls and Their Nonpolar Derivatives Using a Binary Solvent Delivery System[a]

Time (min)	Flow rate (ml/min)	% Solvent A	% Solvent B
0.0	1.0	100.0	0.0
10.0	1.0	50.0	50.0
25.0	1.0	50.0	50.0
30.0	1.0	100.0	0.0

[a]Solvent A, 75:25 (v/v) methanol/water; solvent B, ethyl acetate.

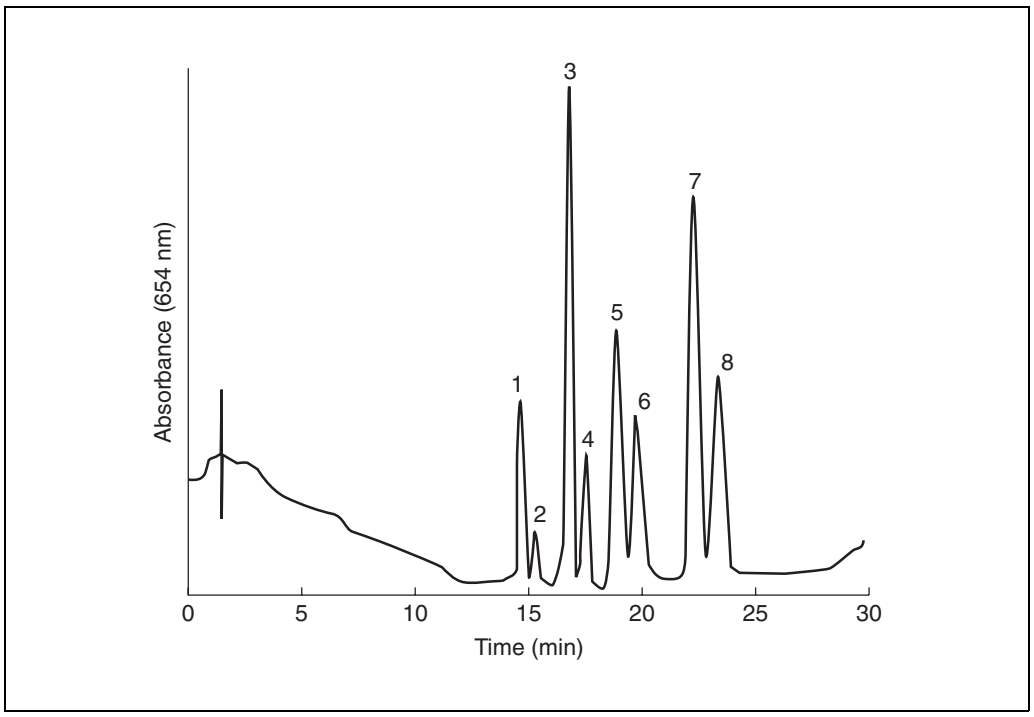

Figure F4.4.1 Typical HPLC chromatogram of eight major chlorophyll derivatives separated using the Basic Protocol. Peak identifications: 1, chlorophyll *b*; 2, chlorophyll *b′*; 3, chlorophyll *a*; 4, chlorophyll *a′*; 5, pheophytin *b*; 6, pyropheophytin *b*; 7, pheophytin *a*; 8, pyropheophytin *a*.

remains a luxury item for this method, which can still be carried out using a simple variable-wavelength detector capable of detection at 654 nm, and authentic standards for comparison and identification.

Baseline drift is a normal effect of gradient delivery (see Critical Parameters and Troubleshooting) and therefore a blank (100% HPLC-grade acetone) should be run and subtracted from the sample, if desired.

14. Run appropriate chlorophyll standards. Calculate chlorophyll concentrations from generated peak integration data according to the response curves for each specific standard.

An example of an HPLC chromatogram obtained using these conditions is given in Figure F4.4.1.

ALTERNATE PROTOCOL

C18 REVERSED-PHASE HPLC SEPARATION OF POLAR CHLOROPHYLL DERIVATIVES AND Cu^{2+} AND Zn^{2+} PHEOPHYTINS

Polar chlorophyll derivatives and metalloporphyrin derivatives such as Cu^{2+} and Zn^{2+} pheophytins can also be analyzed by C18 reversed-phase HPLC. Appropriate standards must be used; see *UNIT F4.2* for polar chlorophyll derivatives, or see Support Protocol 2 for Cu^{2+} and Zn^{2+} pheophytin standards. Gradient solvent conditions and flow rates are given in Tables F4.4.3 and F4.4.4. Otherwise, the separation is performed as described for chlorophylls and nonpolar derivatives (see Basic Protocol). Using this method, separation of polar chlorophyll derivatives can be achieved in 20 to 25 min, and separation of the metalloporphyrin derivatives in 20 to 25 min. Examples of chromatograms obtained for polar derivatives, Zn^{2+} pheophytins, and Cu^{2+} pheophytins are shown in Figures F4.4.2, F4.4.3, and F4.4.4, respectively.

Table F4.4.3 Linear Gradient Conditions for Separation of Polar Chlorophyll Derivatives[a]

Time (min)	Flow rate (ml/min)	% Solvent A	% Solvent B
0.0	1.3	100.0	0.0
6.0	1.3	100.0	0.0
7.0	1.5	70.0	30.0
10.0	1.5	70.0	30.0
11.0	1.5	60.0	40.0
12.0	1.5	50.0	50.0
13.0	1.5	0.0	100.0
24.0	1.5	0.0	100.0
26.0	1.5	100.0	0.0

[a]Solvent A, 15:65:20 (v/v/v) ethyl acetate/methanol/water; solvent B, 60:30:10 (v/v/v) ethyl acetate/methanol/water.

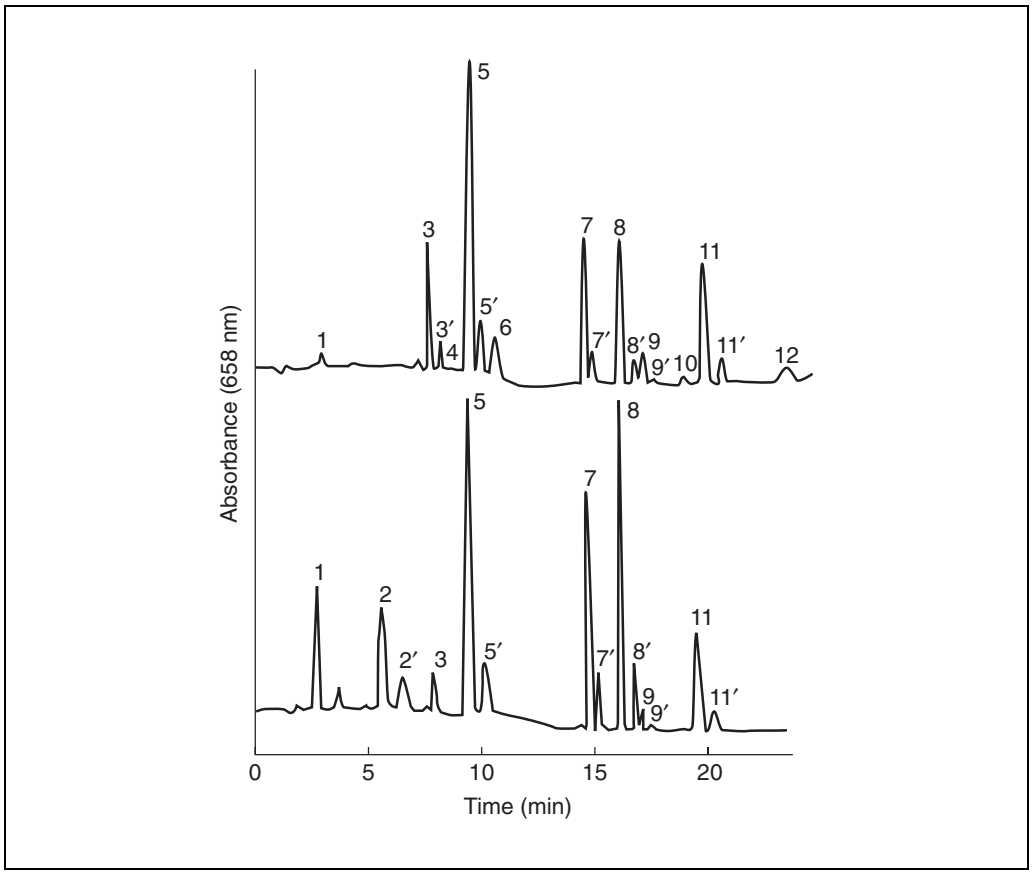

Figure F4.4.2 HPLC chromatogram of chlorophyll derivatives separated using the Alternate Protocol. Peak identifications: 1, chlorophyllide *b*′; 2, chlorophyllide *a*; 2′, chlorophyllide *a*′; 3, pheophorbide *b*; 3′, pheophorbide *b*′; 4, pyropheophorbide *b*; 5, pheophorbide *a*; 5′, pheophorbide *a*′; 6, pyropheophorbide *a*; 7, chlorophyll *b*; 7′, chlorophyll *b*′; 8, chlorophyll *a*; 8′, chlorophyll *a*′; 9, pheophytin *b*; 9′, pheophytin *b*′; 10, pyropheophytin *b*; 11, pheophytin *a*; 11′, pheophytin *a*′; 12, pyropheophytin *a*. Reproduced from Canjura et al. (1991) with permission from the Institute of Food Technologists.

Table F4.4.4 Linear Gradient Conditions for Separation of Cu^{2+} and Zn^{2+} Pheophytins[a]

Time (min)	Flow rate (ml/min)	% Solvent A	% Solvent B
0.0	2.0	55.0	45.0
15.0	2.0	50.0	50.0
16.0	2.0	55.0	45.0

[a]Solvent A, 75:25 (v/v) methanol/water; solvent B, ethyl acetate.

Table F4.4.5 Step Gradient Conditions for C18 Column Clean-Up

Time (min)	% Methanol	% Water	% Ethyl acetate
0.0	75.0	25.0	0.0
15.0	100.0	0.0	0.0
30.0	50.0	0.0	50.0
45.0	0.0	0.0	100.0
75.0	50.0	0.0	50.0
90.0	100	0.0	0.0
105.0	75.0	25.0	0.0

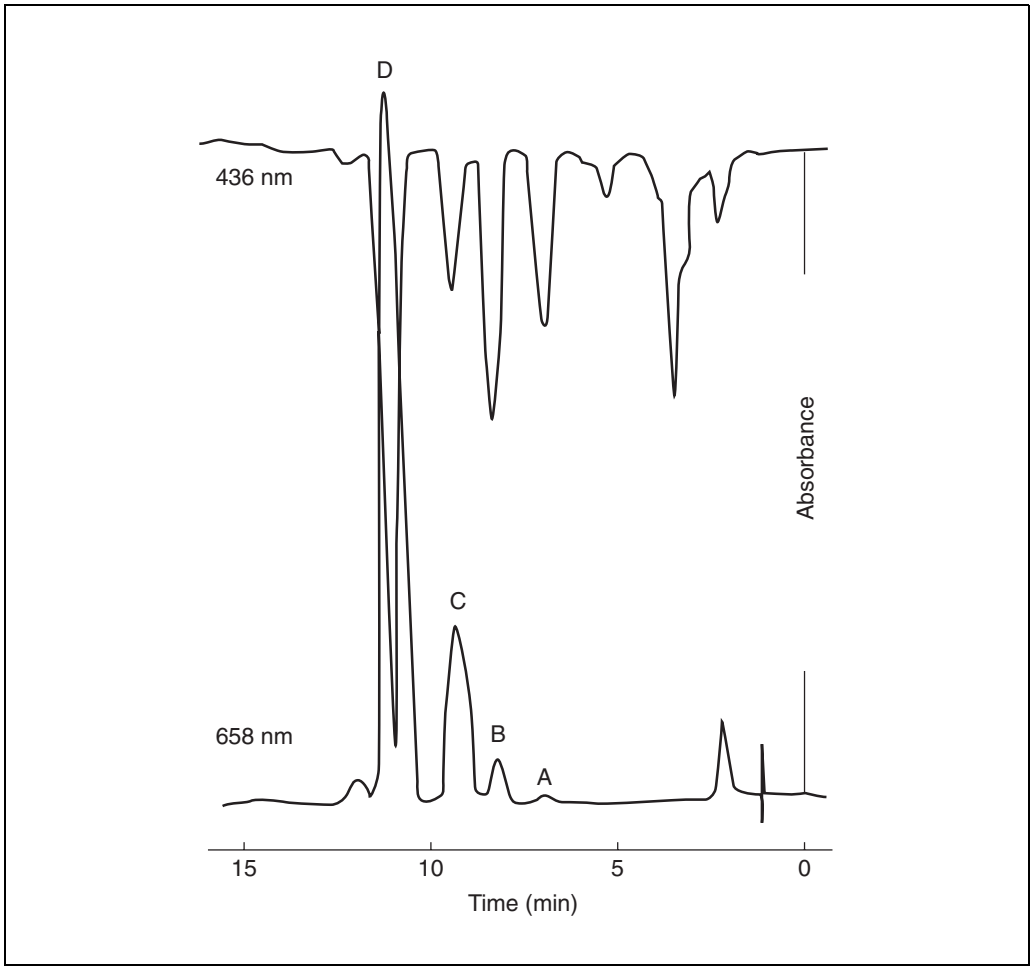

Figure F4.4.3 HPLC chromatogram of Zn^{2+} pheophytins separated using the Alternate Protocol. Peak identifications: A, allomerized Zn^{2+} pheophytin *b*; B, Zn^{2+} pheophytin *b*; C, allomerized Zn^{2+} pheophytin *a*; D, Zn^{2+} pheophytin *a*. Reproduced from Schwartz (1984) with permission from Marcel Dekker, Inc.

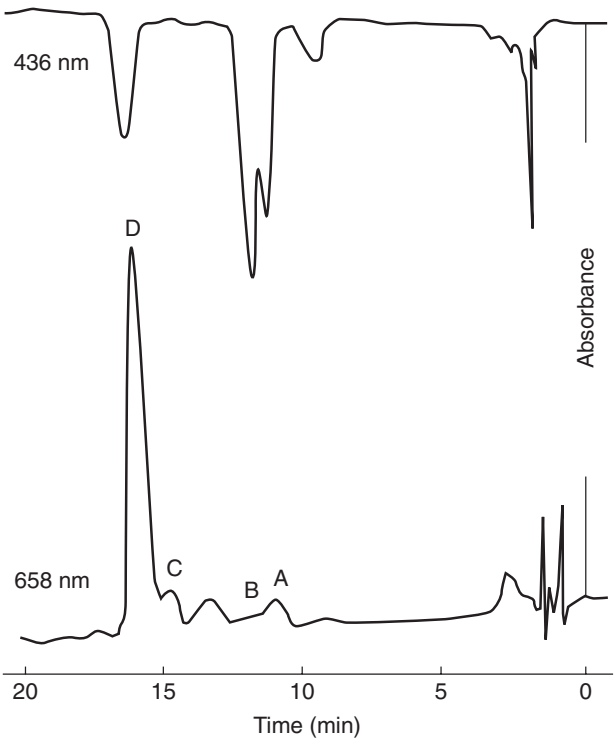

Figure F4.4.4 HPLC chromatogram of Cu^{2+} pheophytins separated using the Alternate Protocol. Peak identifications: A, allomerized Cu^{2+} pheophytin *b*; B, Cu^{2+} pheophytin *b*; C, allomerized Cu^{2+} pheophytin *a;* D, Cu^{2+} pheophytin *a*. Reproduced from Schwartz (1984) with permission from Marcel Dekker, Inc.

C18 COLUMN CLEANING

SUPPORT PROTOCOL 1

Between analyses, and at the end of each day, the HPLC analytical column should be reequilibrated with the appropriate initial gradient conditions until system pressure returns to initial operating conditions. However, on occasion, the analytical column may require a more rigorous cleaning procedure as described below in order to ensure continued optimal performance. Examples of conditions where this may be required are listed in Table F4.4.6.

1. When analysis is complete, allow system to equilibrate analytical column with appropriate initial gradient conditions as stipulated in Table F4.4.1 or Table F4.4.2 for ≥15 min.
2. Program clean-up gradient conditions as described in Table F4.4.5.

   A step gradient is used for this method whereby each of the mobile phase compositions described in Table F4.4.5 is used for 15 min, with the exception of 100% ethyl acetate, which is used for 30 min.

3. Initiate and run the clean-up gradient at a flow rate of 1.0 ml/min.

   The run will take 2 hr.

4. Upon completion of the clean-up gradient, reequilibrate analytical column with appropriate initial gradient conditions for ≥15 min.

   If the column is being prepared for storage, 100% methanol should be used for equilibration for ≥30 min prior to removal and sealing. This will help ensure longer column life.

SUPPORT PROTOCOL 2

SYNTHESIS OF Cu^{2+} AND Zn^{2+} PHEOPHYTIN STANDARDS

The use of appropriate analytical standards is important for successful chromatographic separation, identification, and quantification of chlorophyll derivatives. While chlorophyll *a* and *b* derivatives are readily available commercially (Sigma-Aldrich) both metal-free pheophytins and metalloporphyrin analogs such as Cu^{2+} and Zn^{2+} pheophytins are not. In most instances, these derivatives must be prepared from the parent Mg-chlorophyl standards prior to use. These simple synthesis techniques are based on the work of Schwartz (1984) and are to be utilized for the rapid and efficient preparation of metal-free, Cu^{2+} and Zn^{2+} pheophytin derivatives in quantities appropriate only for analytical implementation.

Additional Materials *(also see Basic Protocol)*

Concentrated hydrochloric acid (HCl; ~37% solution)
HPLC-grade diethyl ether
Zinc chloride
2.5 M $CuCl_2$ in HPLC-grade H_2O
Anhydrous sodium sulfate
Nitrogen gas
Glass vials (≥11 ml; at experimentor's discretion)

1. Dissolve enough crystalline chlorophyll *a* standard in a total volume of 10 ml HPLC-grade acetone to give an absorbance of ~1.0 at 661 nm. Repeat with chlorophyll *b* (646 nm).

   Chlorophyll a and b standards can be purified from plant tissue as outlined in UNIT F4.2. Standards are also available commercially from Sigma-Aldrich.

2. Acidify each solution with 250 µl concentrated HCl.
3. Gently mix for ~10 min.
4. Extract pheophytins with 5.0 ml HPLC-grade diethyl ether. Add 1.0 ml HPLC-grade water to hasten phase separation.
5. Remove residual acid from diethyl ether layer by three successive washings with 10.0 ml water and transfer pigment-rich ether layer to a clean glass vial.
6. Dry pigment-rich diethyl ether layer by passing through approximately 50 to 100 mg of anhydrous sodium sulfate. Remove solvent under a stream of nitrogen.
7. Redissolve pheophytin *a* and *b* extracts in 4.0 ml acetone each.
8. Add 1.0 ml of 2.5 M $CuCl_2$ to a 2.0-ml aliquot of each pheophytin extract.
9. Add 0.3 g zinc chloride to a 2.0-ml aliquot of each extract.

   The reactions should go to completion within 30 min.

10. Assay the extent of complex formation and final purity by HPLC (see Alternate Protocol).

    Successful formation of Cu^{2+} or Zn^{2+} pheophytin would result in only one major chromatographic peak (>90% of total peak area). The presence of other peaks most likely results from incomplete complexation or allomerization (oxidation) of the chlorophyll molecules. In these instances further chromatographic purification (based on Alternate Protocol) may be necessary in order to achieve the desired degree of analytical purity.

11. Extract the derivatives as described in steps 4 to 6 and store dried and desiccated between −20° and −80°C until use (up to 2 to 3 months).

    Prior to use as authentic standards, the desired Cu^{2+} and Zn^{2+} pheophytins will have to be purified from other impurities stemming from the synthesis. This can be accomplished by simple preparatory or semipreparatory phase chromatography using the method described (see Alternate Protocol). If only a small amount of sample is required, collection of a few fractions from a separation using a standard analytical column may suffice.

Table F4.4.6 Troubleshooting Guide for HPLC of Chlorophylls

Observed problem	Possible cause	Solution
Erratic baseline signal	Incorporation of air in mobile phase	Properly degas solvents as instructed by manufacturer
	Equilibration time too short	Allow 60 min for equilibration
Baseline drift	Normal effect of gradient delivery	Subtract blank from baseline
	Sample overload	Reduce injection volume
	Buildup of retained compounds on column	Clean column (see Support Protocol 1)
Erratic pressure reading (ΔP >100 psi)	Air trapped in pump head	Purge system as instructed by manufacturer
	Slow solvent leak in system	Check all fittings for leaks; tighten or replace if needed
Large pressure drop (ΔP >200 psi)	Solvent leak in system	Check entire system for leaks, including pump, injector, detector
Large pressure increase (ΔP >200 psi)	System fouling	Replace fouled line or filter with clean part
	Solvents prepared incorrectly	Prepare new solvents
	Precipitation of sample on column	Reequilibrate system and reduce injection volume or dilute sample
	Buildup of retained compounds on column	Clean column (see Support Protocol 1)
	Guard column failure	Replace guard column
	Analytical column failure	Replace analytical column
Precipitation of sample in injection vial	Solvent incompatibility	Reextract sample and dissolve in acetone (*UNIT F4.2*)
	Sample temperature too low	Ensure sample is at ≥20°C
	Analyte too concentrated	Dilute sample
Loss of chromatographic resolution	Buildup of retained compounds on analytical column	Clean column (see Support Protocol 1)
	Improper gradient delivery	Verify that gradient is programmed and working correctly
	Solvents prepared incorrectly	Prepare new solvents
	Injection volume too large or concentration too high	Reduce injection volume
	Column temperature too high	Reduce temperature to ≤25°C
	Degradation of analytical column	Replace analytical column
Chromatographic artifacts and apparent peak splitting	Solvent incompatibility	Reextract sample and dissolve in acetone (*UNIT F4.2*)
	Sample degradation	Carry out all work under subdued light and inject sample immediately upon being redissolved
	Detection at incorrect wavelength	Set detector to 654 nm

COMMENTARY

Background Information

The accurate measurement of chlorophylls has importance for numerous reasons ranging from simple color considerations to medical research. The most practical of these is the assessment of fruit and vegetable color quality, as chlorophylls are known to degrade rapidly when subjected to thermal processing (Schwartz et al., 1981). However, interest has also been sparked by recent literature reports that point to the possible health benefits associated with chlorophyll consumption (Hartig and Bailey, 1998).

Tswett was the first to accomplish chromatography of chloroplast pigments in 1903. Over time, advances have been made leading to the development of HPLC. With HPLC becoming the definitive method by which chlorophyll analysis is presently performed, it is not surprising that numerous methods exist in the literature. Techniques range in application from fruits and vegetables to shipboard seawater analysis. Eskins et al. (1977) developed an excellent method for chlorophyll *a* and *b* analysis in fruit. Mantoura and Llewellyn (1983) analyzed fruits and vegetables for chlorophyll and carotenoids as well as their breakdown products. However, these methods are often found to be complex, labor intensive, and time consuming, with long run times. More recent methods have focused on other pigments as well as chlorophylls. Minguez-Mosquera et al. (1992) described a method to separate chlorophylls and carotenoids from virgin olive oil that quantified seventeen pigments including seven chlorophyll derivatives.

The widespread application of HPLC methodology to chlorophyll analysis demonstrates its flexibility, effectiveness, and reliability. Methods described in this unit are based on the work of Schwartz et al. (1981). This original method allows for resolution of twelve chlorophyll derivatives in 30 min. While minor modifications were made to this method to further simplify the analysis, the final resolution and sensitivity were not compromised. Based on a commercially available reversed-phase column and an aqueous mobile phase, the method can be easily altered for specific separations. This is demonstrated in the Alternate Protocol, where the Basic Protocol was adjusted for the analysis of polar and Cu^{2+}- and Zn^{2+}-containing chlorophyll derivatives. The method for polar derivatives is based on the separation of Canjura and Schwartz (1991), while the method for Cu^{2+} and Zn^{2+} pheophytin analysis is based on Schwartz (1984). Together, these methods allow the analyst to set up a chromatographic system based on a single stationary phase that can easily be modified for flexible and complete analysis of relevant chlorophyll derivatives. When combined with the appropriate extraction methodology, these protocols can be applied to any application relevant to chlorophyll analysis.

Critical Parameters and Troubleshooting

HPLC techniques often come with a list of potential problems. A troubleshooting guide is summarized in Table F4.4.6. One of the most important considerations is to know the limitations of the HPLC system itself. Pressure limits, solvent compatibility, gradient capability, and other parameters should all be confirmed as described in the manufacturer's instructions. However, monitoring the system pressure is a good practice that can allow for an early detection of possible problems. Generally, it is normal for system pressure to fluctuate due to changes in mobile phase composition. One should therefore establish a normal working range for this important parameter. For example, the system pressure in the Basic Protocol may be 1200 psi under initial conditions, drop to 700 psi at 20 min, and then reach 1200 psi just at 30 min. Therefore, the working range of 700 to 1200 psi should be maintained throughout the run. If the pressure increases or drops dramatically beyond this range, maintenance may be necessary (Table F4.4.6).

Anticipated Results

Typical chromatograms from all protocols described in this unit can be seen in Figures F4.4.1 to F4.4.4.

Time Considerations

The overall analysis time is dependent on a few factors, one of which is column length. With a standard analytical column length of 250 mm, run times of 25 to 30 min can be expected. Shorter, 150-mm columns may be used to shorten the run time; however, some resolution can be lost when using this approach. One must also account for preparation time for HPLC analysis. This includes steps such as sample preparation, instrument equilibration, and data analysis. This generally takes 1 hr per sample for instrumental and data analysis, plus ~1 hr

for each full day's analysis for initial instrument equilibration. Finally, be sure to allocate time for sample preparation (*UNIT F4.2*).

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Key References

Schwartz et al., 1981. See above.

The original method described in the Basic Protocol and modified for the Alternate Protocol. Information regarding sample preparation is also described.

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Mass Spectrometry of Chlorophylls

Mass spectra of chlorophylls and their derivatives have been obtained using a variety of desorption ionization methods including laser desorption (Tabet et al., 1985), matrix-assisted laser desorption/ionization (MALDI; Liu et al., 1999), field desorption (Dougherty et al., 1980), plasma desorption (Hunt et al., 1981), and fast atom bombardment (FAB; van Breemen et al., 1991a; Hyvarinen and Hynninen, 1999; Teng and Chen, 1999). Classical ionization techniques such as electron impact and chemical ionization have been less useful for the analysis of these thermally labile compounds. Liquid chromatography/mass spectrometry (LC/MS) has been applied to the on-line separation of chlorophylls and their degradation products, first using continuous-flow FAB mass spectrometry (van Breemen et al., 1991b) and subsequently using electrospray ionization (ESI; Zissis et al., 1999) and atmospheric pressure chemical ionization (APCI; Airs and Keely, 2000; Verzegnassi et al., 2000). FAB and APCI have been used for ionization of chlorophylls followed by tandem mass spectrometric analysis (MS/MS; van Breemen et al., 1991a; Airs and Keely, 2000). Since FAB, ESI, and APCI LC/MS have been the most widely used mass spectrometric techniques for the analysis of chlorophylls, protocols for these approaches are described in detail below.

FAST ATOM BOMBARDMENT (FAB), LIQUID SECONDARY ION MASS SPECTROMETRY (LSIMS), AND CONTINUOUS-FLOW FAB OF CHLOROPHYLLS

BASIC PROTOCOL 1

FAB and LSIMS are matrix-mediated desorption techniques that use energetic particle bombardment to simultaneously ionize samples like carotenoids and transfer them to the gas phase for mass spectrometric analysis. Molecular ions and/or protonated molecules are usually abundant and fragmentation is minimal. Tandem mass spectrometry with collision-induced dissociation (CID) may be used to produce abundant structurally significant fragment ions from molecular ion precursors (formed using FAB or any suitable ionization technique) for additional characterization and identification of chlorophylls and their derivatives. Continuous-flow FAB/LSIMS may be interfaced to an HPLC system for high-throughput flow-injection analysis or on-line LC/MS.

Materials

Matrix:
3-nitrobenzyl alcohol (for static FAB or LSIMS)
0.5% (w/v) glycerol dissolved in the mobile phase (for continuous-flow FAB or LSIMS)

Chlorophyll sample (1 to 100 mg/liter; 2 to 200 μM; see *UNIT F4.2* for extraction protocols) dissolved in volatile organic solvent such as acetone, diethyl ether, or ethyl acetate (store in airtight glass vial)

HPLC mobile phase solvent (e.g., ethyl acetate, methanol, water, and/or acetone; for continuous-flow FAB/LSIMS LC/MS)

Mass spectrometer or tandem mass spectrometer (JEOL, Micromass, MAT from ThermoFinnigan) equipped with direct insertion probe and fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS); for LC/MS or flow injection using continuous-flow FAB, mass spectrometer must be equipped with continuous-flow ionization source

Microsyringe (for static FAB or LSIMS; 10-μl Hamilton or equivalent)

Contributed by Richard B. van Breemen
Current Protocols in Food Analytical Chemistry (2001) F4.5.1-F4.5.9

HPLC setup (for continuous flow FAB/LSIMS LC/MS; also see *UNIT F4.4*)
including:
Syringe pump or HPLC pump capable of flow rates of 1 to 10 µl/min
Reversed-phase (typically C18) column (since flow rate into continuous-flow FAB source must be <10 µl/min, capillary column must be used or flow must be split post column)

Additional reagents and equipment for HPLC of chlorophylls (*UNIT F4.4*)

For static FAB or LSIMS analysis

1a. Load 0.5 to 1 µl of the 3-nitrobenzyl alcohol matrix onto the sample probe using a microsyringe.

The 3-nitrobenzyl alcohol matrix is typically stable at room temperature for ~3 months. Older solutions begin to turn yellow as they decompose and should be replaced.

Although other matrices such as glycerol may be used, 3-nitrobenzyl alcohol is the most effective matrix that has been reported for static FAB or LSIMS of chlorophylls.

2a. Load 1 µl of the chlorophyll sample onto the surface of the liquid matrix. Let solvent evaporate.

3a. Insert probe through the vacuum interlock into the ion source of the mass spectrometer.

4a. Turn on the FAB or LSIMS beam and record the positive ion mass spectrum over the range *m/z* 100 to 1000. If desired, eliminate the 3-nitrobenzyl alcohol ion at *m/z* 154 by scanning from *m/z* 200 to 1000.

Static FAB/LSIMS provides a continuous signal for chlorophylls and their derivatives so that exact mass measurements may be carried out to determine elemental compositions, or tandem mass spectra following CID may be recorded.

For continuous-flow FAB/LSIMS LC/MS

1b. Set up HPLC system using reversed-phase (i.e., C18) column and interface with mass spectrometer according to the manufacturer's instructions.

UNIT F4.4 provides details on HPLC of chlorophylls.

2b. Run mobile phase containing 0.5% glycerol matrix through system such that the flow rate into the mass spectrometer does not exceed 10 µl/min.

During LC/MS, the glycerol matrix is included in the mobile phase and does not interfere with reversed-phase chromatography or in-line absorbance detection of chlorophylls. Matrices other than glycerol might be suitable for continuous-flow FAB of chlorophylls, but none have been reported.

3b. Load chlorophyll sample onto the HPLC injector as indicated by the manufacturer. See *UNIT F4.4* for additional details.

Since microbore HPLC columns are usually used for continuous flow FAB, the sample volume is usually 1-5 µl.

4b. Acquire data by turning on the FAB or LSIMS beam and recording positive ion mass spectra over the range *m/z* 200 to 1000.

The scan rate should not exceed 8 sec/scan so as to preserve chromatographic resolution. Ideally, at least 8 mass spectra should be acquired per chromatographic peak. All mass spectra are stored separately in chronological order using LC/MS software, provided by the instrument manufacturer. These mass spectra may be retrieved and viewed later or used to produce computer-reconstructed ion chromatograms.

LC/MS USING ATMOSPHERIC PRESSURE CHEMICAL IONIZATION (APCI) AND ELECTROSPRAY IONIZATION (ESI)

APCI and ESI are atmospheric pressure interfaces between HPLC systems and mass spectrometers. These interfaces serve the dual functions of removing the carrier solvent from the HPLC eluate and ionizing the analyte. During APCI, the HPLC eluate is sprayed through a heated capillary into a chamber with a counter-current or cross-flow of heated nitrogen gas that facilitates the evaporation of solvent. A corona discharge in this chamber forms a steady state of solvent ions that ionize the analyte through collision processes similar to those in classical chemical ionization. During ESI, the HPLC eluate is sprayed through a capillary held at high potential relative to the surrounding chamber. As a result, electrospray droplets are smaller than those formed during APCI, and analyte ions are formed directly from the charged spray. LC/MS analyses of chlorophylls and related compounds have been reported using APCI (Airs and Keely, 2000; Verzegnassi et al., 2000) and ESI (Zissis et al., 1999).

Materials

Chlorophyll sample (1 to 100 mg/liter; 2 to 200 µM; see *UNIT F4.2* for extraction protocols) dissolved in volatile organic solvent such as acetone, diethyl ether, or ethyl acetate (store in airtight glass vial)

HPLC mobile phase: may include combinations of ethyl acetate, methanol, diethyl ether, methyl *tert*-butyl ether, acetonitrile, water, and/or acetone (all are compatible with APCI and ESI)

HPLC system interfaced to a mass spectrometer or tandem mass spectrometer equipped with APCI or ESI (e.g., Agilent, Mircromass, ThermoFinnigan)

C18 reversed-phase column for HPLC separations of chlorophylls: narrow-bore (2.1-mm i.d.) column at flow rate of 50 to 300 µl/min without splitting the flow, or analytical column (4.6-mm i.d.) at 1 ml/min with post-column solvent splitting of 5:1 (200 µl/min entering the mass spectrometer) for APCI; electrospray interfaces are available for use without solvent splitting over all flow rates from nl/min to 1 ml/min

UV/VIS absorbance detector (single wavelength or diode array) placed in-line between HPLC column and mass spectrometer for additional characterization of eluting chlorophylls (optional)

Additional reagents and equipment for HPLC of chlorophylls (*UNIT F4.4*)

1. Set up HPLC system and begin running mobile-phase solution at a flow rate of 200 µl/min into the APCI or ESI mass spectrometer.

   UNIT F4.4 provides details on HPLC of chlorophylls.

2. Inject a 1- to 5-µl aliquot of the chlorophyll solution onto the HPLC system using a microsyringe with a manual injector or using an autoinjector under computer control.

   HPLC separation of the chlorophyll mixture should be carried out using reversed-phase (usually C18 columns) as described in UNIT F4.4.

   Mass spectrometric or MS/MS detection of chlorophylls using APCI requires ~5 pmol of each compound per analysis (Airs and Keely, 2000).

3. *Optional:* Direct the eluate from the HPLC column through a UV/VIS absorbance detector prior to the mass spectrometer for additional sample characterization.

4. Divert the solvent front from the HPLC column, containing salts and unretained material, to waste instead of the mass spectrometer in order to minimize fouling of the LC/MS interface.

   LC/MS systems may be equipped with programmable switching valves for this purpose.

5. Acquire LC/MS data by scanning the range *m/z* 200 to 1000 so that at least 8 mass spectra are acquired per chromatographic peak.

   Mass spectra recorded using LC/MS software may be displayed individually, signal averaged, and background subtracted. Furthermore, these data may be used to plot computer-reconstructed selected ion or total ion chromatograms.

COMMENTARY

Background Information

FAB and LSIMS

In widespread use since 1982 (Barber et al., 1982), FAB and LSIMS are matrix-mediated techniques. The most effective matrix for static FAB/LSIMS analysis of chlorophylls and their derivatives is 3-nitrobenzyl alcohol (van Breemen et al., 1991a), whereas glycerol provides adequate sensitivity and a more robust system during continuous-flow FAB/LSIMS (van Breemen et al., 1991b). Ionization and desorption of the chlorophyll analyte occur together during the bombardment of the matrix by fast atoms (or ions) to produce molecular ions, $M^{+\cdot}$, and protonated molecules, $[M+H]^+$.

FAB ionization has been used in combination with LC/MS in a technique called continuous-flow FAB LC/MS (van Breemen et al., 1991b). Although any standard HPLC solvents may be used, including ethyl acetate, methanol, and water, the mobile phase should not contain nonvolatile additives such as phosphate or Tris buffers. Volatile buffers such as ammonium acetate are compatible. The low flow rate of continuous-flow FAB/LSIMS (<10 µl/min) is the primary limitation of this technique.

APCI and ESI

Since APCI and ESI interfaces operate at atmospheric pressure and do not depend upon vacuum pumps to remove solvent vapor, they are compatible with a wide range of HPLC flow rates. HPLC methods that have been developed using conventional detectors such as UV/VIS, IR, or fluorescence are usually transferable to LC/MS systems without adjustment. However, the solvent system should contain only volatile solvents, buffers, or ion-pair agents to reduce fouling of the mass spectrometer ion source. In the case of chlorophyll solvent systems, isocratic and gradient combinations of methanol, acetonitrile, water, acetone, and/or ethyl acetate have been used for APCI or ESI LC/MS. Unlike continuous-flow FAB/LSIMS, no sample matrix is necessary.

The APCI interface uses a heated nebulizer to form a fine spray of the HPLC eluate and to facilitate solvent evaporation. In addition, a cross-flow of heated nitrogen gas is used to

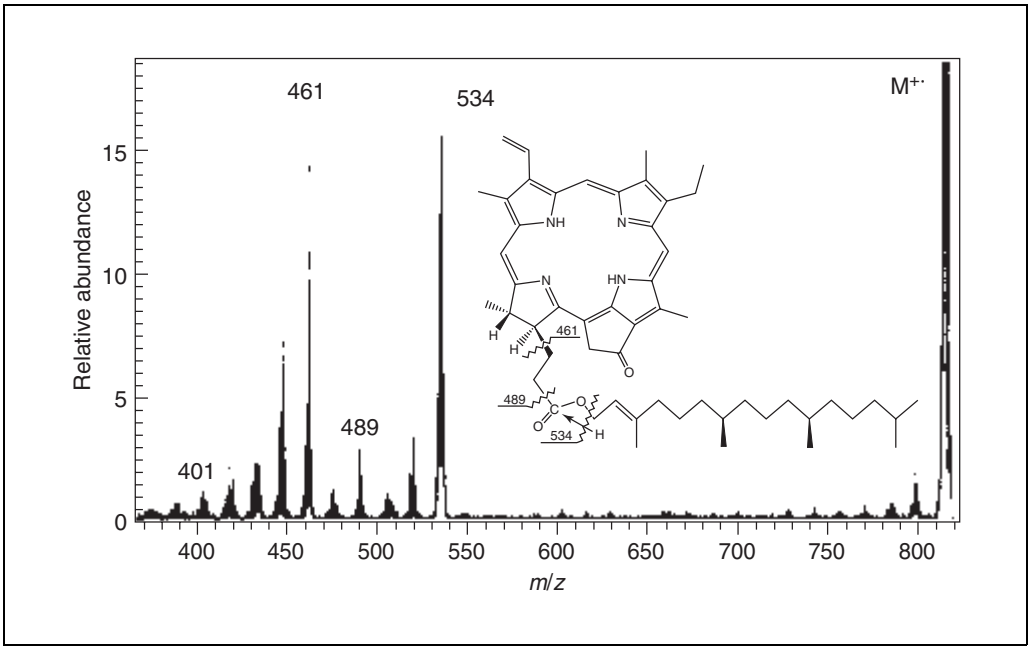

Figure F4.5.1 Positive ion fast atom bombardment (FAB) with collision-induced dissociation (CID) tandem mass spectrum of pyropheophytin *a* isolated from spinach leaves. The FAB matrix was 3-nitrobenzyl alcohol.

complete the evaporation of solvent from the droplets. The resulting gas-phase sample molecules are ionized by collisions with solvent ions, which are formed by a corona discharge in the atmospheric pressure chamber. Chlorophyll ions formed during APCI consist of cationized species such as protonated molecules, $[M+Na]^+$ and $[M+K]^+$, and positive and negative molecular ions. The relative abundance of each type of ion depends upon the solvent used, the presence of proton donors or acceptors, the levels of alkali metal ions in the sample and mobile phase, and the ion source parameters. Ions are then drawn into the mass spectrometer analyzer for measurement. A narrow opening between the mass spectrometer analyzer and the ion source helps the vacuum pumps to maintain very low pressure inside the analyzer while the APCI source remains at atmospheric pressure.

During ESI, the HPLC eluate is sprayed through a capillary electrode at high potential (usually 2000 to 7000 V) to form a fine mist of charged droplets at atmospheric pressure. As the charged droplets are electrostatically attracted towards the opening of the mass spectrometer, they encounter a cross-flow of heated nitrogen that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. ESI of chlorophylls can produce molecular ions as well as cationized species such as protonated molecules and $[M+Na]^+$ and $[M+K]^+$ (Zissis et al., 1999). The relative abundance of the cationized species depends upon the concentration of proton-donating species and trace amounts of salts in the mobile phase.

Both APCI and ESI are compatible with the same HPLC columns and solvent systems used for chlorophyll analysis. However, the main advantage of APCI compared to electrospray for chlorophyll analysis is the linearity of the detector response, which is more than two orders of magnitude larger for APCI. This suggests that APCI LC/MS might be preferred to ESI LC/MS for quantitative analysis of chlorophylls.

Critical Parameters and Troubleshooting

Chlorophylls are stabilized in vivo within chloroplasts in a complex environment of lipids, proteins, and other compounds. Once extracted from the biological matrix, chlorophylls are easily oxidized upon exposure to air and/or light to form allomers and other degradation products (*UNIT F4.1*). Therefore, care should be taken to minimize the exposure of chlorophyll samples to light and air. For example, sample

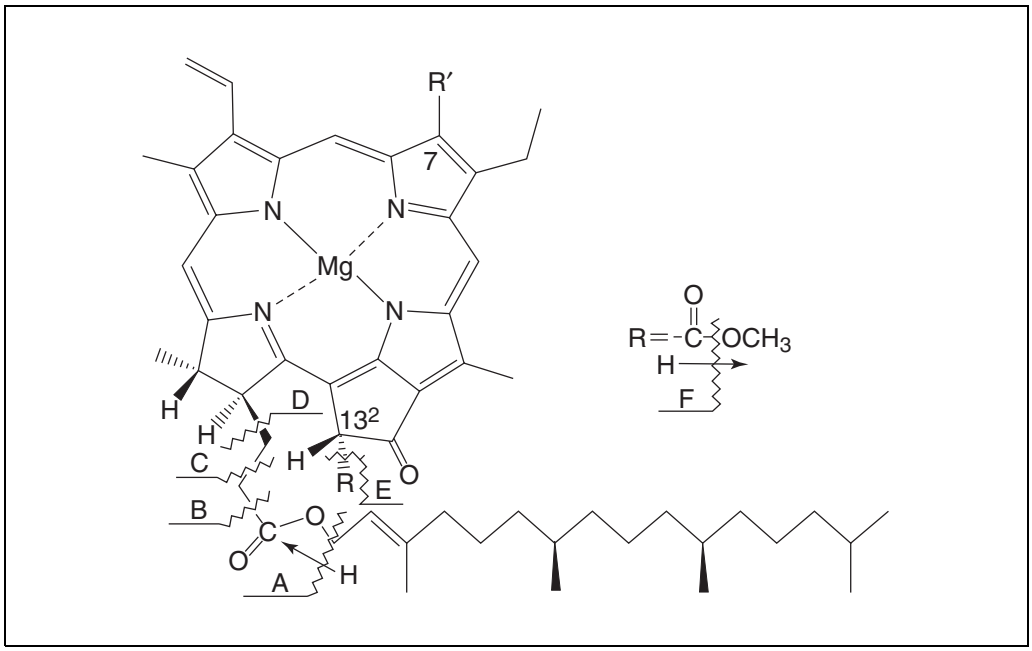

Figure F4.5.2 Mass spectrometric fragmentation scheme for chlorophylls *a* and *b* and their derivatives. Chlorophyll *a*, R = $–COOCH_3$, R′ = $–CH_3$; chlorophyll *b*, R = $–COOCH_3$, R′ = –CHO; pyropheophytin *a*, –Mg +2H, R = H, R′ = $–CH_3$; pyropheophytin *b*, –Mg +2H, R = H, R′ = –CHO; pheophytin *a*, –Mg +2H, R = $–COOCH_3$, R′ = $–CH_3$; pheophytin *b*, –Mg +2H, R = $–COOCH_3$, R′ = –CHO; chlorophyllide *a*, –phytyl chain, R = $–COOCH_3$, R′ = $–CH_3$; chlorophyllide *b*, –phytyl chain, R = $–COOCH_3$, R′ = –CHO; pheophorbide *a* (chlorophyllide *a* –Mg +2H); pheophorbide *b* (chlorophyllide *b* –Mg +2H).

vials or flasks containing chlorophylls should be amber, or else wrapped in an opaque covering to minimize the exposure of the contents to light. Furthermore, containers should be filled with inert gas such as nitrogen or argon. When opening sample vials, the room should be dark or dimly lit.

The solvents that are used for extraction of chlorophylls from leaves or other biological samples are similar to those used for HPLC (*UNIT F4.4*). For example, leaves are usually ground in the presence of acetone to extract chlorophylls. After filtration, the solvent is evaporated in vacuo or under a stream of nitrogen (but not air, to avoid oxidation). One additional purification step prior to HPLC (applicable to both Basic Protocols 1 and 2) is to dissolve the chlorophyll residue in hexane and then wash the hexane with water/methanol (1:1; v/v). The hexane may be removed prior to HPLC or simply diluted with mobile phase prior to analysis.

Anticipated Results

Molecular ions and protonated molecules of chlorophylls and related compounds will be formed during static FAB/LSIMS when using 3-nitrobenzyl alcohol as the matrix. Alternatively, a glycerol matrix will facilitate the formation of protonated molecules for chlorophylls and their derivatives during continuous-flow FAB. Using static FAB/LSIMS, sample ions will be produced continuously for several minutes until the sample or the matrix is consumed. During this time, exact mass measurements, high-resolution measurements, and tandem mass spectrometric measurements may be carried out to characterize the chlorophyll sample.

As an example, the tandem mass spectrum with CID of pyropheophytin *a* is shown in Figure F4.5.1. Pyropheophytins can be formed from chlorophylls as a result of cooking or canning of food products. In this case, the molecular ion of *m/z* 812 was obtained using positive ion static FAB, fragmented using CID, and the resulting tandem mass spectrum was recorded using a magnetic sector mass spectrometer. Fragment ions were observed that confirm the presence of the phytyl chain (*m/z* 534, 489, and 461). The sites of cleavage within pyropheophytin *a* during CID are indicated in Figure F4.5.1. These and other types of fragment ions that are characteristic of chlorophylls and their derivatives are illustrated in the fragmentation scheme shown in Figure F4.5.2. The fragment ion of type A, $[M-278]^+$, corresponds

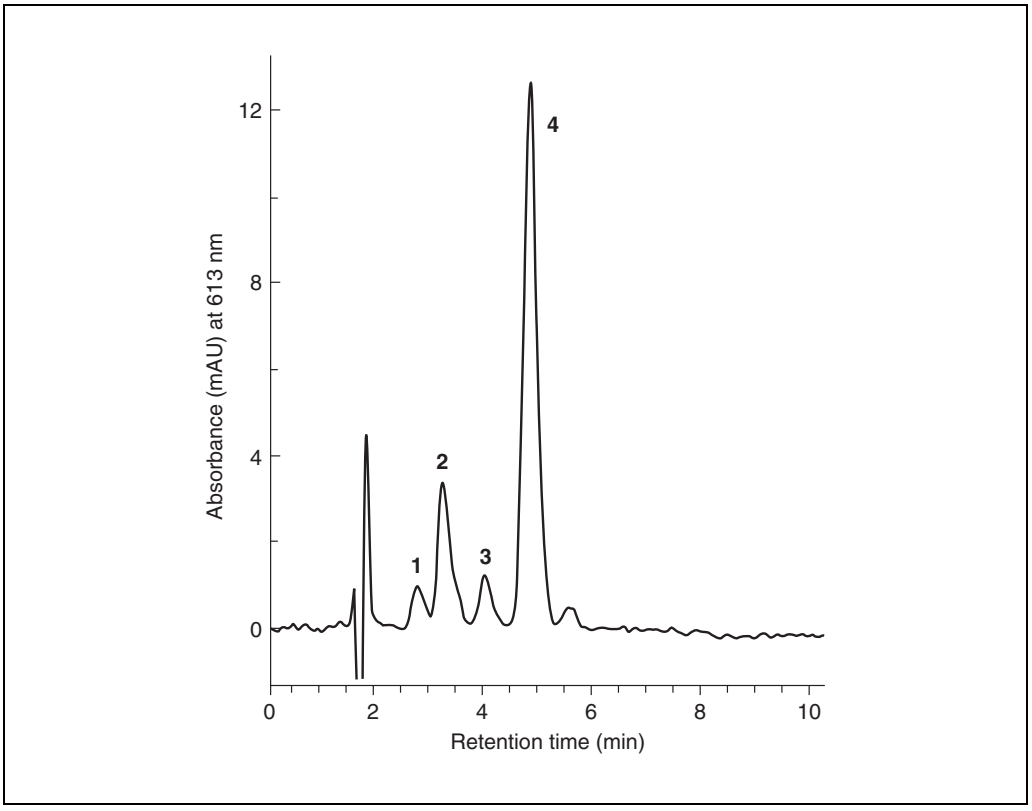

Figure F4.5.3 UV/VIS absorbance chromatogram at 613 nm of an acetone extract of spinach leaves recorded during UV/VIS LC/MS. Examples of APCI mass spectra recorded during this analysis are shown in Figure F4.5.4.

to elimination of the phytyl chain with a transfer of a hydrogen from the ion to the leaving group. Whereas ions of types A, B, and D are formed by loss of the phytyl chain and cleavage at various points along the side chain on C17, the absence of fragment ions of types E and F from the tandem mass spectrum of pyropheophytin *a* shows that this chlorophyll derivative does not contain a β-keto ester group or a methyl group at position C13[2] (R in Figure F4.5.2).

Tandem mass spectrometry following CID of molecular ions or cationized molecules may be used to enhance the formation of structurally significant fragment ions and to eliminate matrix ions and other contaminating ions from the mass spectrum. Although the example shown in Figure F4.5.1 was obtained using FAB, any other ionization method suitable for chlorophylls, such as ESI or APCI, may be used. Fragment ions provide information about chlorophyll functional groups and help to distinguish chlorophyll *a* from chlorophyll *b* and from related pheophytins, chlorophyllides, pheophorbides, and pyropheophytins. Chlorophyll *a* may be distinguished from chlorophyll *b* by the presence of a methyl group instead of a formyl group at position C7 (R′ in Figure F4.5.2). This results in a molecular weight difference of 14 (chlorophyll *a* weighs 892 and chlorophyll *b* weighs 906 mass units). Additional details regarding structure determination of chlorophylls and their derivatives using MS/MS with CID may be found in van Breemen et al. (1991a).

The UV/VIS LC/MS analysis of an acetone extract of spinach leaves is shown in Figure F4.5.3 and Figure F4.5.4. Reversed-phase C18 chromatography was used with UV/VIS photodiode array absorbance detection followed on-line by positive ion APCI mass spectrometry. The mobile phase consisted of a 20-min linear gradient from methanol to methanol/methyl *tert*-butyl ether (91:9; v/v). The visible absorbance chromatogram at 613 nm for

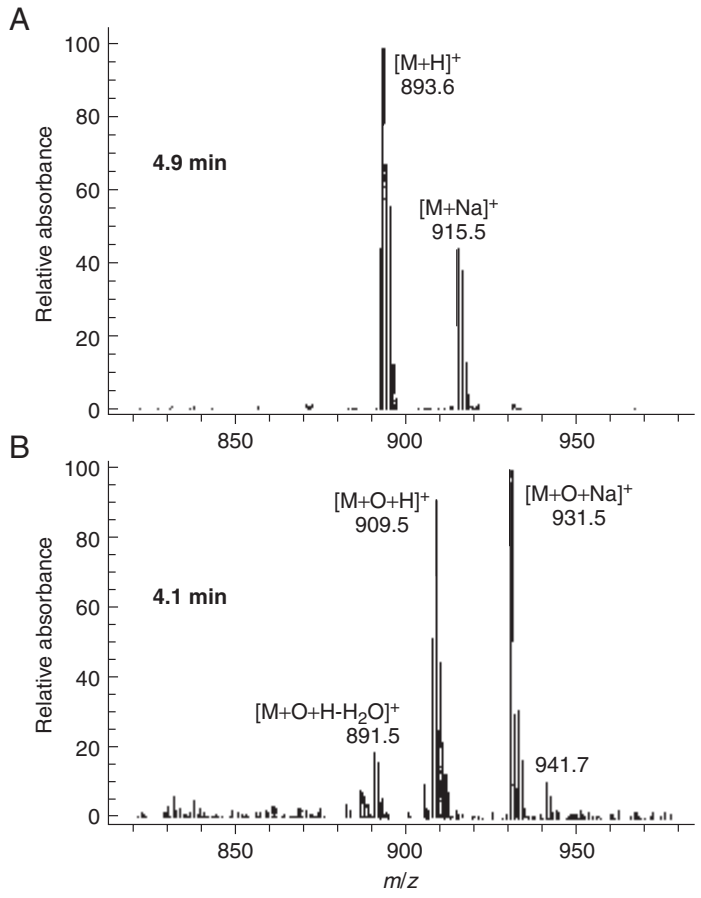

Figure F4.5.4 Positive ion APCI mass spectra of (**A**) chlorophyll *a* and (**B**) an oxidation product of chlorophyll *a*, which eluted at 4.9 and 4.1 min, respectively, during the UV/VIS LC/MS chromatogram shown in Figure F4.5.3.

this analysis is shown in Figure F4.5.3. Four peaks were detected instead of the expected two peaks for chlorophylls *a* and *b*. Diode array detection indicated that all four peaks in Figure F4.5.3 corresponded to chlorophylls. However, mass spectrometric detection was necessary to distinguish these compounds from each other. Post-column addition of 0.5% acetic acid (so that the mobile phase contained 0.05% acetic acid) was used to enhance the abundance of protonated molecules of the chlorophylls in the positive ion APCI mass spectra. Based on the mass spectra and diode array absorbance spectra, peaks 2 and 4 were identified as chlorophyll b and chlorophyll *a*, respectively, whereas peaks 1 and 3 were oxidation products of chlorophylls *b* and *a*, respectively, weighing an additional 16 mass units. The positive ion APCI mass spectra for chlorophyll a and its oxidation product (peaks 3 and 4) are shown in Figure F4.5.4.

For additional characterization of these chlorophylls, high-resolution, exact mass measurements could be carried out to confirm their elemental compositions using a magnetic sector mass spectrometer, a quadrupole time-of-flight hybrid mass spectrometer, or a Fourier transform ion cyclotron resonance mass spectrometer. In addition, tandem mass spectrometry could be used to obtain additional information regarding the structures of these compounds. A quadrupole mass spectrometer was used to obtain the data shown in Figure F4.5.4. Although an excellent instrument for high-throughput LC/MS analyses, a single quadrupole analyzer is not capable of MS/MS nor does it offer sufficient precision and resolution for exact mass measurements. Finally, HPLC could be used to purify sufficient quantities of each peak for NMR characterization. Typically, at least 1000-fold more sample is required for NMR than for mass spectrometry. Since this process would require the investment of considerably more time and sample handling, it would result in even more sample degradation.

Note that the extraction process used in the example discussed here resulted in the formation of measurable oxidation products. When this extract was reanalyzed just 24 hr later without storage under an inert atmosphere or in a freezer, no chlorophyll *a* or *b* could be detected. In summary, UV/VIS LC/MS or LC/MS/MS provides the most rapid and sensitive approach to characterizing and identifying chlorophylls and their degradation products.

Time Considerations

Since mass spectrometry is a rapid analytical technique, the sample throughput will be ~20 per hour when using FAB/LSIMS and >60 per hour when using flow injection analysis with APCI or ESI. The rate-limiting step for FAB/LSIMS is the time (1 to 2 min) required to introduce the direct insertion probe through the vacuum interlock. During LC/MS and LC/MS/MS, the slow step is the time required for chromatographic separation.

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Key References

van Breemen et al., 1991a. See above.

The most complete reference of tandem mass spectra for chlorophylls and chlorophyll derivatives including pheophytins, chlorophyllides, pheophorbides, and pyropheophytins.

Verzegnassi et al., 2000. See above.

Class characteristic fragment ions of chlorophylls and related chlorins are summarized for both positive and negative ion APCI. Detailed instrument parameters for LC/MS using APCI are described.

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Overview of Color Analysis

APPEARANCE AND COLOR

The measurement of color is a young science, and is recognized as part of a larger field of study known as appearance science. Appearance can be defined as the phase of visual experience by which things are recognized. There are several appearance modes to identify the situation when a light source or object is recognized. The object mode encountered daily is the most familiar mode. It consists of a person observing an object illuminated by a light source. People make appearance judgments all day long without being aware of how an object is easily recognized as dull or glossy, transparent or opaque. Appearance technology classifies objects based on their distribution of incident light. Appearance attributes can be divided into two main categories—those related either to spectral (color) or to geometric (spatial) light distribution.

A strict definition of color includes (1) the object appearance that depends on light, object, and observer, and (2) the visual perception described with color names. Color is a primary attribute of appearance and it can be quantified. The measurement of color is known as colorimetry. The colorimetric principles associated with the response of the normal eye are important when reviewing color analysis. The eye-brain combination is sensitive, flexible, and able to analyze data at high speeds. Color scientists developed color (spectral) and gloss (geometric) scales founded on human color perception. They studied the response of an observer to light distributed by an object. The phenomenon of color results from this interaction of light, object, and observer. The brain processes the light distributed by the object and reaching the eye. An object has no inherent color, and so it may be stated that color exists only in the mind of the viewer. A group of trained observers will differ in their judgment and description of a perceptible color difference. The sensation of color varies even for observers with normal color vision, not to mention those without normal color vision. Approximately 8% of the male population and 0.5% of the female population are color-defective. Other factors can also influence the color experience. When identified, they lead to a method for color evaluation.

The eye, as amazing as it is, cannot measure color quantitatively. Color-order systems have been developed to specify color based on a space with coordinates. Color can be presented as an arrangement of three dimensions within a color space. One dimension relates to a lightness attribute and the other two are chromatic attributes, referred to as hue and chroma (or saturation). The human observer is not equally

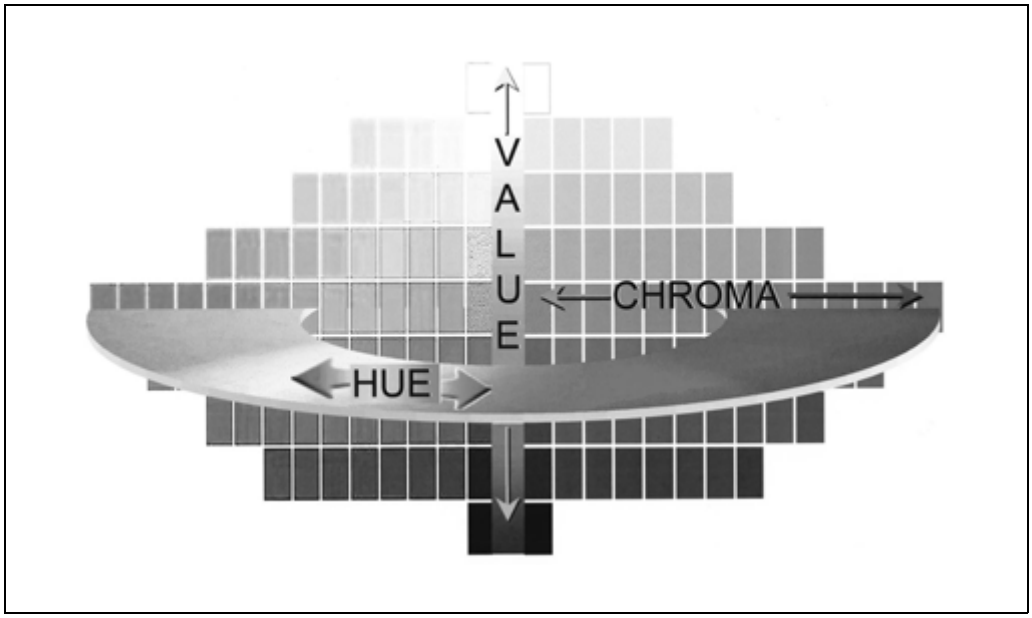

Figure F5.1.1 The Munsell system describes color in terms of hue, value, and chroma. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

Contributed by Kevin Loughrey

Current Protocols in Food Analytical Chemistry (2002) F5.1.1-F5.1.13

sensitive to the three dimensions. Hue is generally the most critical, followed by chroma, and finally lightness. Munsell is a well-known color-order system that specifies surface color and has found wide acceptance in a diversity of color applications. Munsell identifies the hue, value (lightness), and chroma (saturation) of a color using color chips of equal visual spacing (Fig. F5.1.1.). The chips are arranged on pages in a book. All the chips on a single page are the same hue, but they vary in value and chroma. The observer chooses the chip that best matches the color of the object being evaluated. The chip selected will have a notation that identifies the assigned hue, value, and chroma. Visual color standards like Munsell have long been used in various food applications to specify color.

The subject of color, as it relates to the human eye and brain, is complex and fascinating. Overall, there are many factors that can affect the perceived color of a surface. Models like Munsell use an alphanumeric notation to identify the dimensions for a color and its location within the color space. A Munsell notation (hue, value, and chroma) depends on a subjective visual judgment made by a human observer. There are other color-order systems that specify color by objective three-dimensional instrument numbers. The goal of these systems is to establish a reasonable method that measures objects for color and color difference using an instrument. The value in such a method is the potential for the instrument to confirm subjective visual judgments. Successful color analysis concludes with agreement between visual assessment and instrument numbers.

VISUAL COLOR EVALUATION AND THE OBSERVER SITUATION

The experiential nature of color can be understood by examining the basic situation that results in the experience of color. The sensation of color requires three components: a light source, an object that is illuminated, and the eye-brain as a detector. Colorimetry simply refers to this three-way relationship as the observer situation. A complete understanding of this vital relationship is needed for success in the visual and instrumental evaluation of color. The regular practice for visual color appraisal must be considered carefully. Any apparatus used for examining color, such as a light booth, should incorporate illumination and viewing controls. Visual evaluations need to be precise and reproducible. A change in the viewing conditions will have a great impact on the

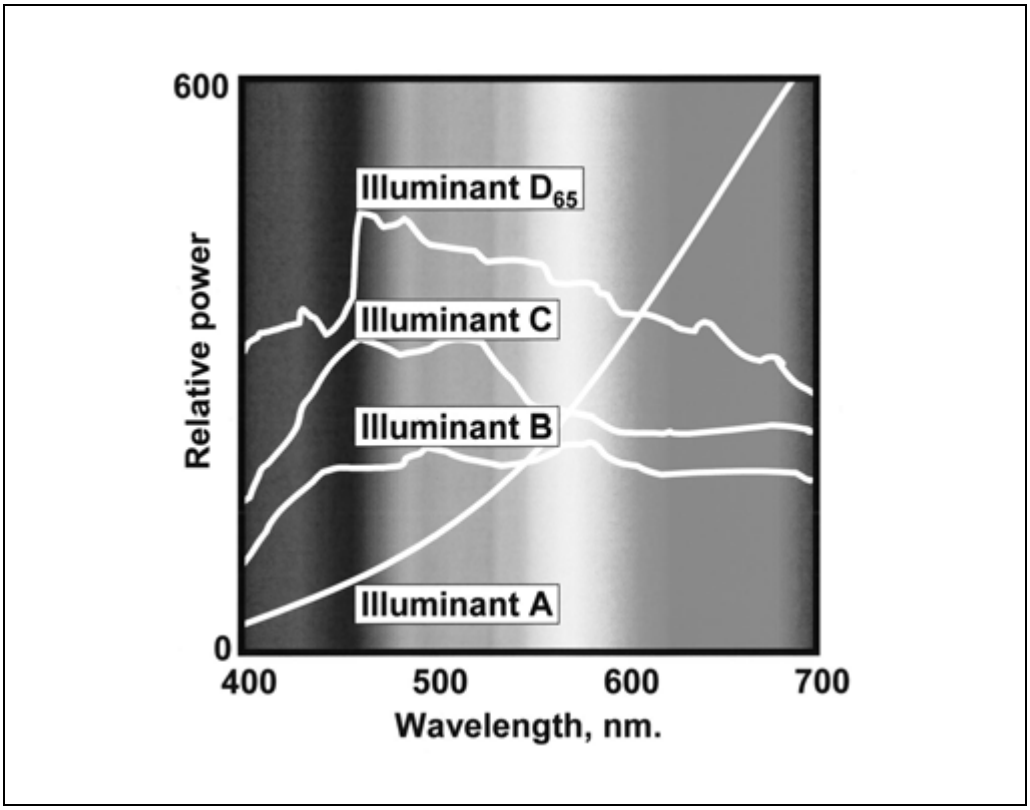

Figure F5.1.2 The spectral power distribution curves of standard CIE illuminants. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

results of any visual appraisal. Standardized conditions for a viewing environment will greatly improve the inspection and approval of color. A number of components are critical to the success of visually judging color:

the spectral quality of the light source;
the level of illumination;
the geometric conditions;
the surround and ambient field;
the observer response functions.

A written method would define the viewing conditions. They must be the same for all observers when judging color. An excellent reference for guidance in visual color appraisal is the published standard ASTM D 1729-96 (ASTM, 2000).

The Light Source

The light source is the first important element of the observer situation. The statement has been made that without light there is no color. Sir Isaac Newton conducted experiments with an optical prism and sunlight to confirm that white light is composed of spectral colors. The human response to color is limited to the visible spectrum of light, containing wavelengths between 380 and 770 nm. The wavelengths are associated with colors, beginning with violet at the short end of the spectrum, through indigo, blue, green, yellow, orange, and finally red at the long end. The nanometer (nm) is the accepted unit of length for light waves. The light is referred to as an illuminant when color is computed using data from a spectrophotometer. Standard illuminants for color measurement were first established in 1931 by the CIE (Commission Internationale de l'Éclairage, or The International Commission on Illumination). The CIE is the main international organization concerned with color and color measurement. Illuminants A (incandescent), B (noon sunlight), and C (overcast daylight), were recommended by the CIE for use in colorimetry. Illuminants are specified by their spectral energy distributed across the visible spectrum. Each illuminant has its own spectral power distribution curve (Fig. F5.1.2), which supports accurate identification. The curve consists of energy plotted against wavelength in nanometers. When measuring color with a spectrophotometer, the standard illuminant table is a selection in the instrument firmware or software. In 1965, the CIE proposed a D series of illuminants to better represent natural daylight. Today the illuminant most widely used in color measurement is D_{65} (average daylight). Standard illuminant tables provide a method to quantify the light as the first element of the observer situation.

The Object

The second element of the observer situation is the object. Incident light is modified when it interacts with an object. There are four main effects (Fig. F5.1.3) that may occur from this interaction:

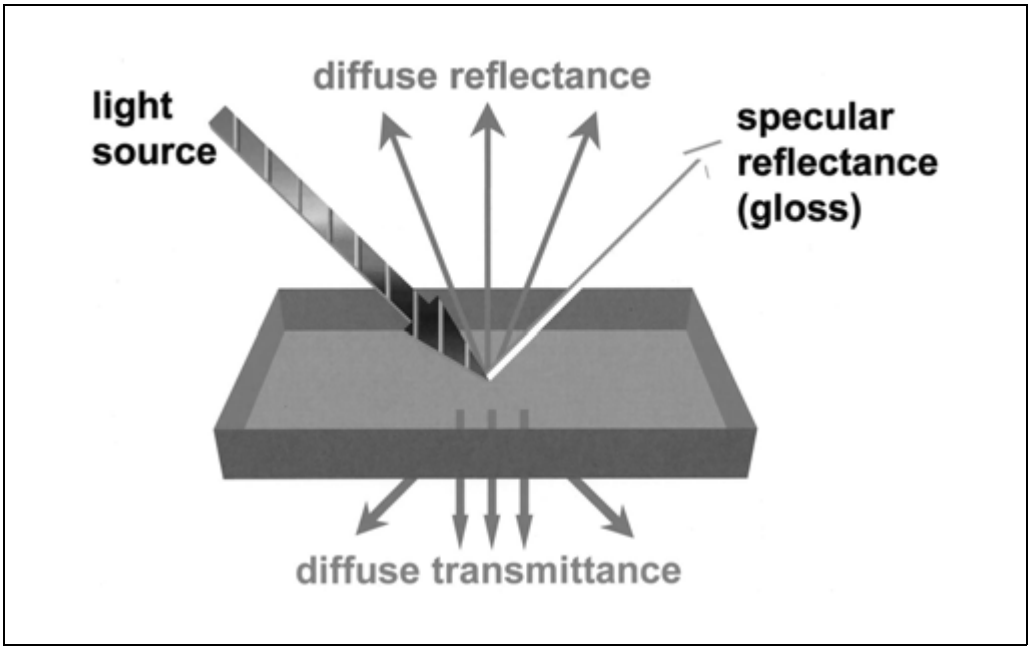

Figure F5.1.3 The interaction of light with an object modifies the light in a variety of ways. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

1. Specular reflection (gloss) at the surface.
2. Selective absorption within the object.
3. Diffuse reflection by scattering in the object.
4. Regular transmission through the object.

Selective absorption is the process of absorbing only certain wavelengths of light. It is the primary basis for the color of an object. A yellow color results mainly from the blue wavelengths being absorbed and the green, yellow, and red wavelengths being reflected or transmitted. In this example, the blue wavelengths of light are absorbed and never reach the eye and brain to trigger a response. The light reflected or transmitted by the object finally reaches the eye-brain of the observer and produces the perception of color. Color instruments are designed to measure this reflected or transmitted light and convert the physical data to three-dimensional numbers, corresponding to perceived color. Instrument color numbers are primarily used to assess quality in the food industry. Food color measurements determine the color quality of food ingredients. They are also used to monitor color quality during processing and storage.

Analytical instruments and techniques are employed in food technology. Certain analytical spectrophotometers are designed to measure percent absorbance at selected wavelengths. A procedure for color analysis may stipulate the measurement of the light-absorbing properties of a food. The analytical methods of color analysis differ from the colorimetric techniques. The summary of color analysis presented here is confined to the colorimetric measurement of food. A color spectrophotometer measures the light reflected or transmitted by an object employing a dispersing element such as a grating to measure at wavelength intervals of 10 or 20 nm. Data from the spectrophotometer are plotted against wavelength and displayed as a spectral curve (Fig. F5.1.4). The spectral curve is like a fingerprint of the object measured. The color of an object may be suggested if the shape of its curve relates to the curves of known colors. The spectral data for the object quantify it as the second element of the observer situation. The illuminant table first selected can now be combined with the data for the object.

The Human Observer

During the last eighty years, research has been conducted within the color community to test the response of the human eye to color stimuli. Scientists knew it was possible to match the color sensation by designing an experiment based on the mixing of three colored lights. W.D. Wright in 1928 and J. Guild in 1931 conducted the most important of these experiments. Their two experiments were inde-

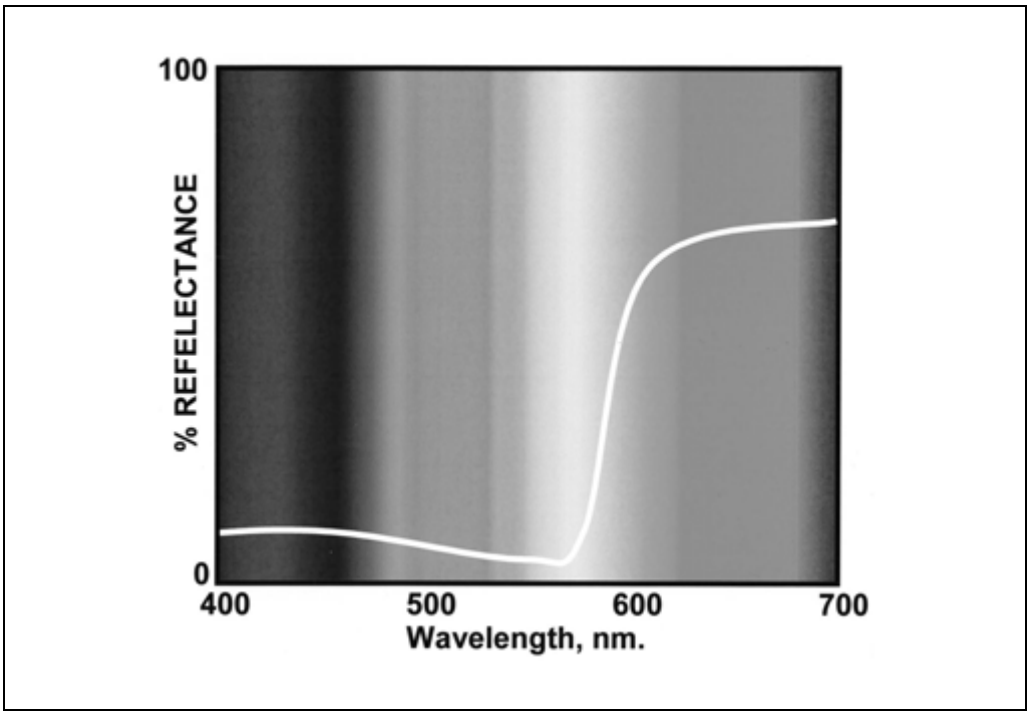

Figure F5.1.4 The spectral reflectance curve of a red object plots reflectance at each wavelength. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

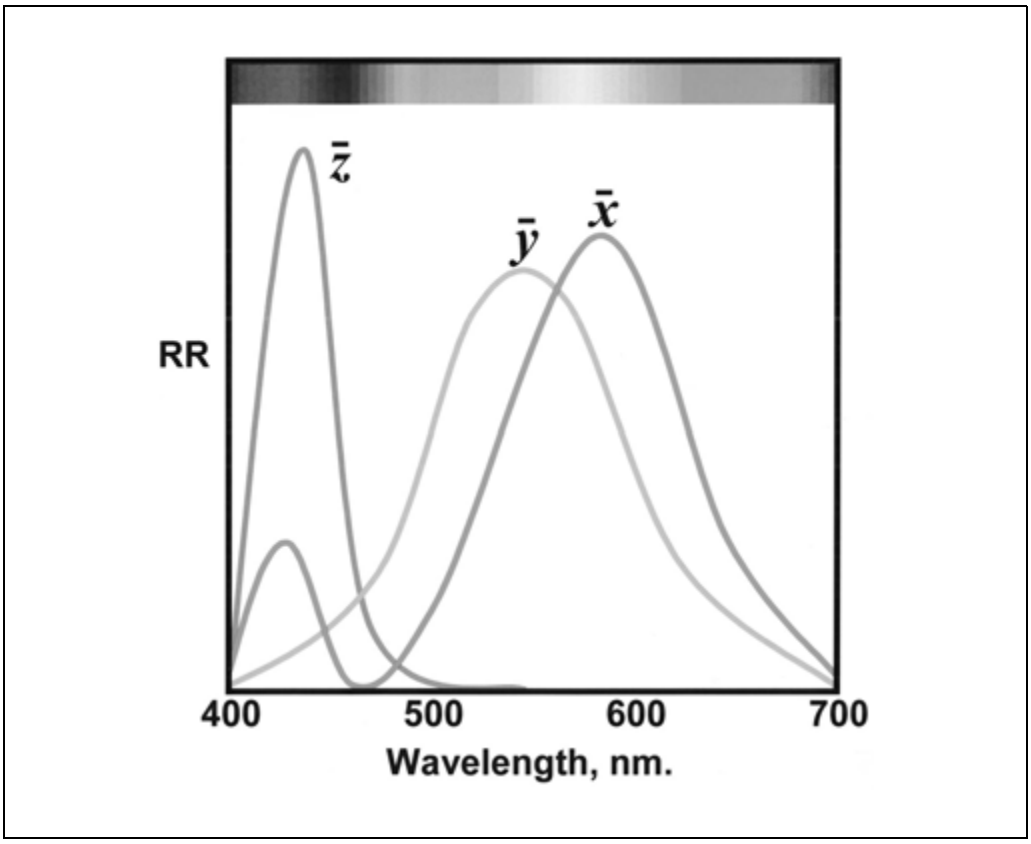

Figure F5.1.5 The 1931 CIE 2° Standard Color Observer established colorimetry. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

pendent of each other, using different observers and different primary lights. The field of view in the experiments was 2° (similar to viewing a dime at arm's length) and was selected to involve the area of the eye known as the fovea. The fovea, located in the central region of the retina, is where the red-, green-, and blue-sensitive cones are concentrated. The cones are the receptor cells in the eye that respond to light at different wavelengths. In both experiments, normal observers visually matched monochromatic (single-wavelength) lights by mixing together three primary lights (red, green, and blue). The data curves from the experiments corresponded to the spectral response of the average human eye. The curves from the two experiments were different because Wright and Guild did not use the same set of primary wavelengths for their three lights.

The CIE transformed the original experimental curves from Wright and Guild into more useful functions that would facilitate the identification of color stimuli by numbers. The three functions resulting from the transformation represent the color-matching response functions of the average observer with normal color vision. The observer functions were standardized and adopted worldwide in 1931 as the CIE 2° Standard Color Observer (Fig. F5.1.5). Standard Observer tables are available for the original 1931 2° Observer and the more recent 1964 10° Observer. The 10° Observer was developed as an improvement of the original 2° Observer and is widely used today in many color applications. The human observer is now quantified as the third element of the observer situation. The observer joins the light and the object so the elements of the observer situation are quantified across the spectrum:

the light source as a spectral power distribution curve;

the object as a spectral reflectance or transmittance curve;

the observer as the spectral response curves.

Light, object, and observer as curves can be combined to compute numbers that agree with perceived color.

MEASURING TRISTIMULUS VALUES

The development of the observer response functions is the foundation for color measured by an instrument. The Standard Observer established a recognized method for converting

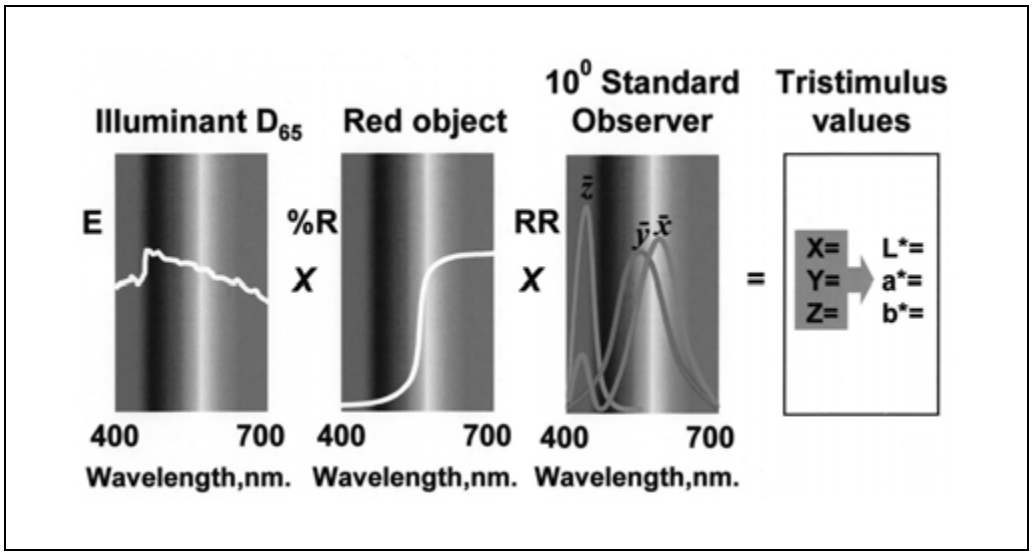

Figure F5.1.6 Light, object, and observer are combined to calculate CIE values *X*, *Y*, and *Z*. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

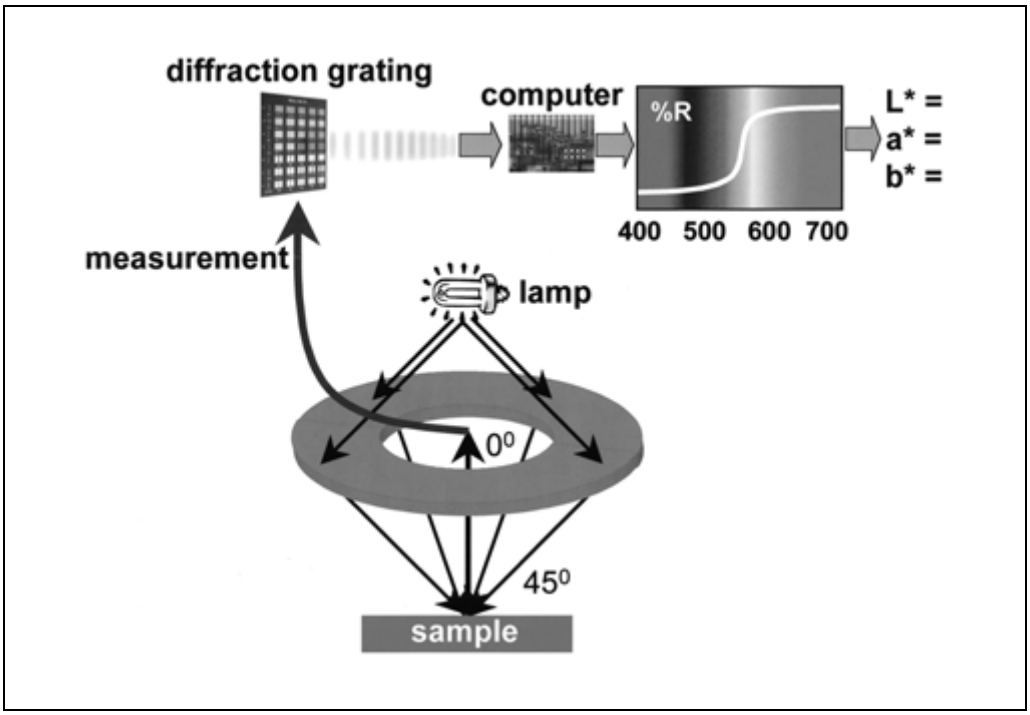

Figure F5.1.7 A color spectrophotometer measures the reflectance of a sample to compute color. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

the spectral curve of any object into three numbers known as the CIE tristimulus values *X,Y,Z* (Fig. F5.1.6). The spectrophotometer measures the object for spectral data and the computer converts the data into the tristimulus values for a selected illuminant and observer (Fig. F5.1.7). The spectrophotometer has been referenced because the measured spectral data can be converted into tristimulus numbers that correspond to perceived color. There are other instruments that measure color, but their design is different from a spectrophotometer.

Tristimulus colorimeters are used to measure color in certain food applications. They combine light source, filters, and photodetectors to reproduce the CIE Standard Observer response functions (Fig. F5.1.8). Colorimeters, having broad band-pass filters, do not measure the spectral data. Without spectral data they cannot offer the choice of either observer or

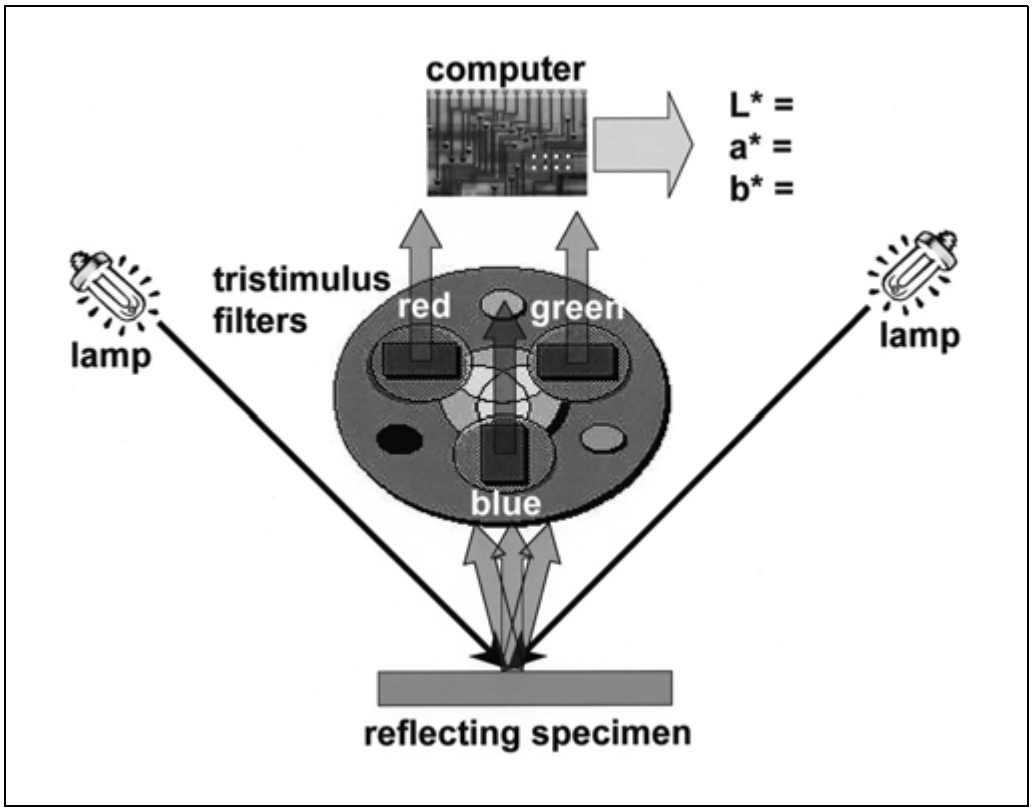

Figure F5.1.8 A colorimeter reproduces the eyes' response for one illuminant and one observer. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

various illuminants such as A (incandescent) and F_2 (cool white fluorescent). Traditionally, their output has been limited to Illuminant C (daylight) for the 2° Standard Observer. Color spectrophotometers do not have the limitations of colorimeters because they measure the percent reflectance or transmittance (spectral data) wavelength by wavelength across the visible spectrum. The spectral data can be converted to tristimulus data for any of the illuminant-observer combinations selected. It should be noted that both spectrophotometers and tristimulus colorimeters are primarily utilized as instruments to measure the color difference between a standard and a trial, rather than as absolute devices.

CIE *XYZ* TO CIELAB

The CIE color-order system is the foundational color scale for all instrumental color scales. However, the *X,Y,Z* values are difficult to correlate with the perceived color of an object. In fact, the CIE scales were not intended for specifying the color of objects, nor the color difference between objects. Also, CIE scales do not have equal visual spacing for all colors. The same numerical color difference between colors does not equate to the same visual difference for all colors. Just a few years following the adoption of the CIE system, alternate color systems became available. The new scales were created to overcome the known inadequacies of the CIE scales when measuring the colors of objects. Equations were developed that transformed the CIE data. The alternate color scales were intended to be more uniform in their visual spacing, but did use the CIE system as the base. In reality, these more uniform color spaces were at best an improvement over the CIE system.

One particular transformation of the CIE system was based on the Hering theory of color vision. The premise was that signals from the receptor cones in the eye were coded into light-dark, red-green, and yellow-blue signals as they traveled to the brain. Such color scales are known as opponent-type because they recognize three pairs of opposing signals (Fig. F5.1.9). This means that a color cannot be perceived as red and green at the same time or yellow and blue at the same time. However, a color can be green and yellow or green and blue.

The rectangular coordinates of an early opponent color system (Hunter) labeled the three dimensions of a color as *L,a,b*. The *L* coordinate represents lightness, the *a* coordinate represents redness (+*a*) or greenness (–*a*), and the *b* coordinate represents yellowness (+*b*) or blue-

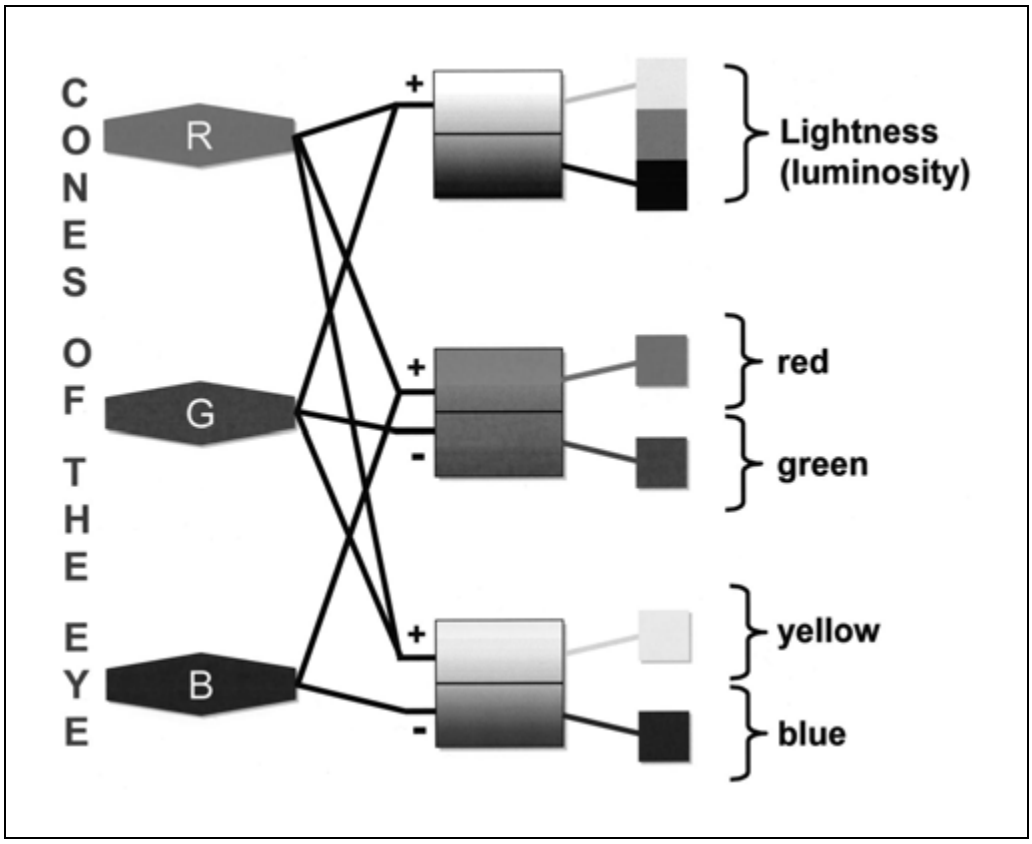

Figure F5.1.9 Hering's theory of color vision stated color is due to three pairs of opposing codes. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

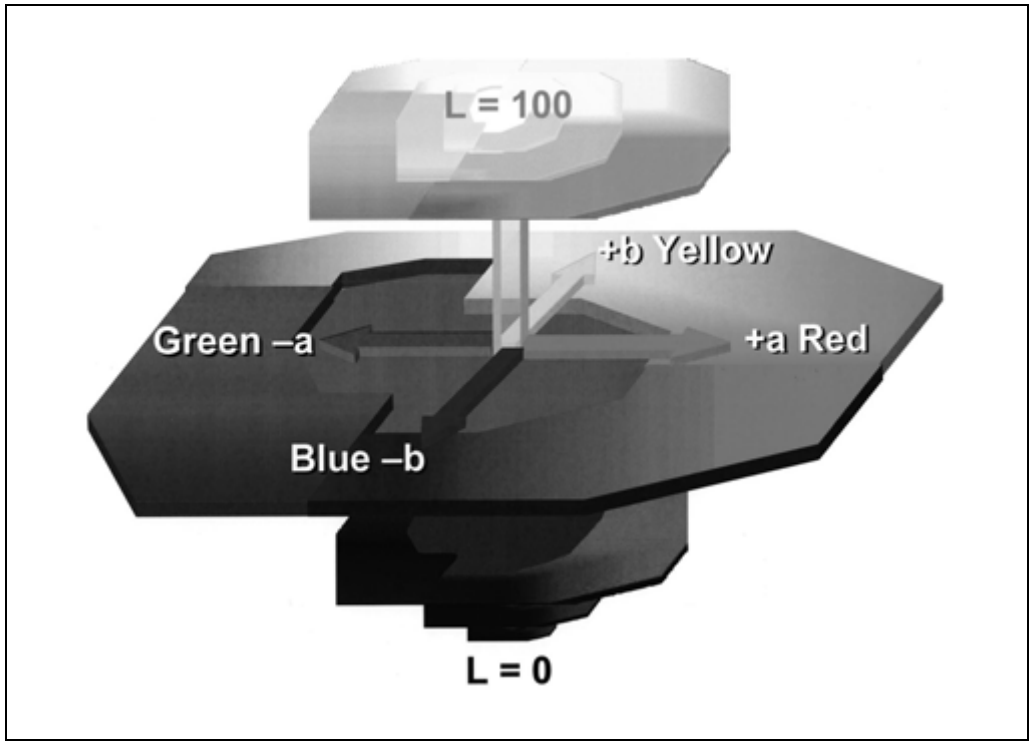

Figure F5.1.10 The Hunter *L*,*a*,*b* color space was designed for measuring color differences. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

ness (–*b*). Using this system, an object measured for color would be assigned an *L,a,b* value to describe the color and specify its location in the three-dimensional color space. The distance between two colors, each having its own location in the color space, relates to a measurable color difference. *L,a,b* numbers correlate with perceived color because the opponent color theory was used to develop the conversion from CIE *X,Y,Z*. The Hunter *L,a,b* color space, published in 1942 (Fig. F5.1.10), became very popular because it described color and color difference in visual terms and was readily available with color instruments (tristimulus colorimeters). Today there are food applications that continue to specify color using the Hunter *L,a,b* color scale.

Work within the color community continued for the ideal, uniform color space. In 1976, the CIE recommended a more nearly uniform color space known as *L*a*b** with the official designation CIELAB. *L*a*b** is an opponent-type color space with rectangular coordinates similar to Hunter *L,a,b*. The *L** indicates lightness (0 to 100), the a* indicates redness (+) and greenness (–), and the *b** indicates yellowness (+) and blueness (–). The limits for *a** and *b** values are around –80 and +80. The two color scales do not correlate and a color located in *L,a,b* space will be in a different location in *L*a*b**. Presently CIELAB is the most widely used color scale across the major industries, but still has limitations.

There has been a growing interest in the food industry for a color space based on a polar model. In 1976 when CIELAB was adopted, the CIE recommended an alternative color scale known as CIELCH or *L*C*H**. Of the three dimensions of color, the hue is the most critical in terms of perceptibility and acceptability for normal color observers. The *L*C*H** color space identifies the hue as one of the three dimensions. A color is located using cylindrical coordinates with *L** being the same as in CIELAB and *C** and *H** computed from *a** and *b**. The coordinates of CIELCH (also see Fig. F5.1.11) are:

*L** = lightness, the same as in *L*a*b**;

*C** = chroma (saturation) is the relation of a color to a neutral gray of the same lightness;

*H** = hue angle is expressed in degrees, with 0° corresponding to +*a** axis (red), then continuing to 90° for the +b* axis (yellow), 180° for –a* (green) and finally 270° for –b* (blue). It is important to realize that now a color is in the same location in either *L*a*b** or *L*C*H** color space. These simply represent two different methods to describe the same location for the same color. *L*C*H** has appeal for food applications because the hue is separately identified and measured.

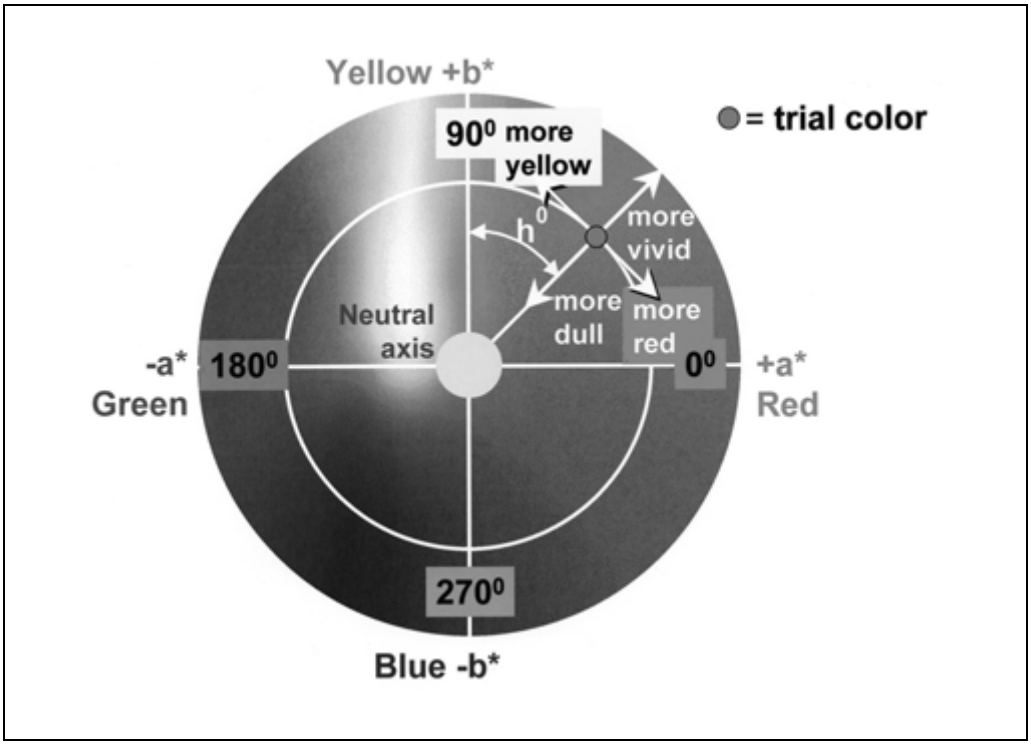

Figure F5.1.11 A sample color located using the polar coordinates of the *L*C*H** color space. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

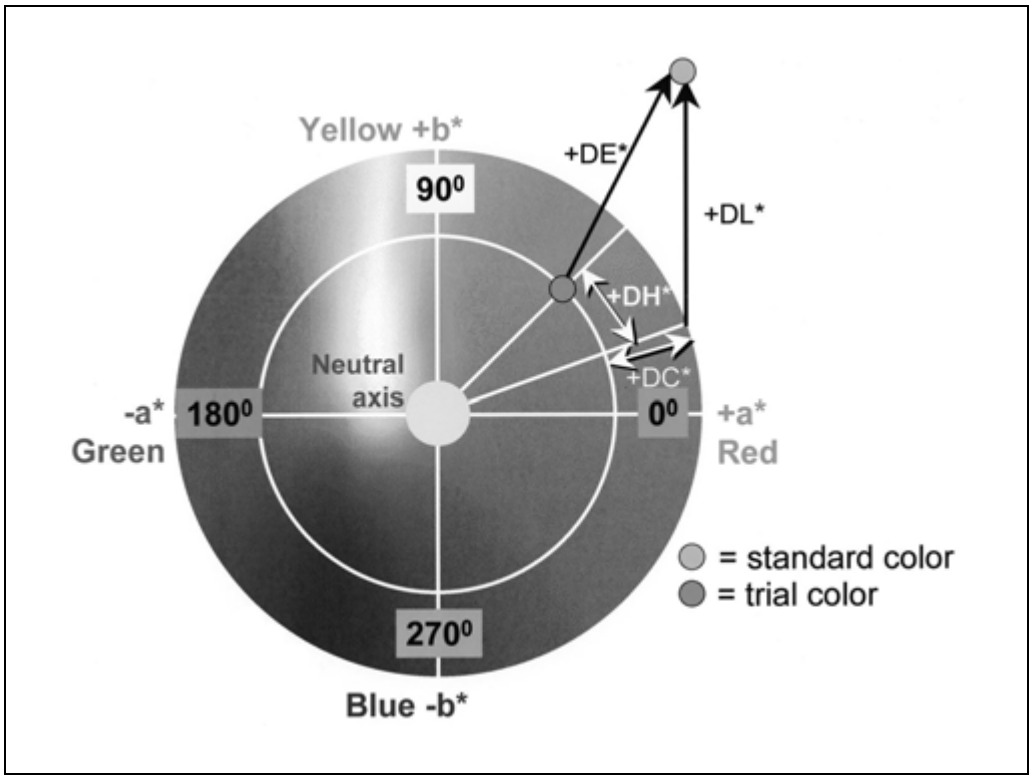

Figure F5.1.12 A difference in hue, chroma, and lightness between a standard and sample. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

COLOR DIFFERENCE EQUATIONS

Many industrial color applications work with a color spectrophotometer to measure CIELAB color differences. CIELAB works well in specifying the color of an object, but color differences calculated with the formula have limited value for pass/fail decisions. These component color differences (*dL**, *da**, *db**) are calculated by simply subtracting the *L*a*b** values for the standard from the *L*a*b** values for the trial. The *dL*, da*, db** values should indicate the direction of color difference from the standard (e.g., lighter or darker). They are commonly used to establish the allowable color limits from a standard. However, in applying these delta differences as limits for color acceptability, they should be adjusted for different colors. CIELAB, like Hunter *L,a,b,* is not a visually uniform color space. The limitations of CIELAB are not well understood throughout industry and the numbers continue to be applied without being adjusted for different colors and products.

For those preferring to express differences in chroma and hue terminology, instead of *da** and *db**, the following terms (also see Fig. F5.1.12) are utilized:

Delta *L** = the lightness difference;

Delta *C** = the chroma angle difference;

Delta *H** = the metric hue difference.

A change in hue of a color is expressed as the distance *DH** (capital *H*) along the chroma arc for the standard. A specific change in hue, *DH**, is larger for a pair of colors (standard and trial) that are far from the neutral axis and smaller for a pair closer to the neutral axis. A negative *DH** indicates the trial is located clockwise in color space from the standard. A positive *DH** means the trial is located counterclockwise from the standard. The direction of the trial from the standard can then be interpreted as a shift in color from the standard. An example of this would be the standard having a hue angle (h°) of 90°, then a positive *DH** means the trial is greener, while a negative *DH** would mean the trial is redder than the standard. The delta differences (*DH** and *DC**) in hue and chroma have been found to agree well with visual perception. However, CIELCH joins Hunter *L,a,b* and CIELAB as a visually nonuniform color space. Different colors require different color acceptability limits.

The Delta *E* (*DE*) is a single number widely used today for color acceptability. A traditional *DE* number is a measure of total color difference and is calculated from the individual com-

ponent differences. When using CIELAB, the color difference equation known as *DE** can be calculated from the component or delta differences. The basic formula for *DE** is:

$DE^* = (dL^{*2} + da^{*2} + db^{*2})^{1/2}$

The *DE* for the Hunter *L,a,b* color scale would be calculated using the same basic equation. However, one *DE* formula cannot be converted into another formula. The Hunter *DE* and the CIELAB *DE** do not correlate, so whatever color difference equation is selected must be specified. Different *DE* calculations have been proposed as a uniform single-number tolerance that could be applied to all colors.

Traditionally the individual delta numbers and the *DE* have served as color tolerances. The difficulty is in the application of color difference numbers when the same measured color difference does not always equate to the same visual difference. A *DE** is a good example, being well established and presently employed

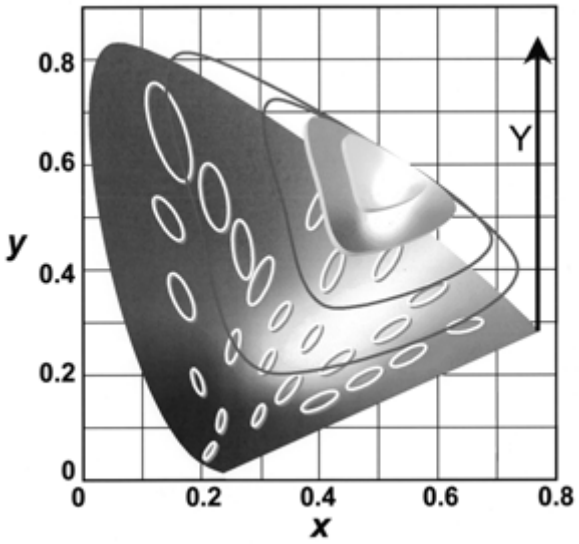

Figure F5.1.13 MacAdam ellipses (MacAdam, 1942) of differing size are plotted within the CIE chromaticity diagram (*x,y*). *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

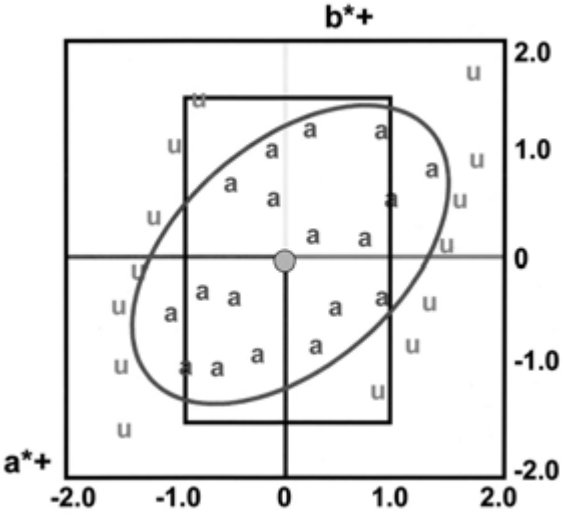

Figure F5.1.14 Elliptical tolerances versus box tolerances. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

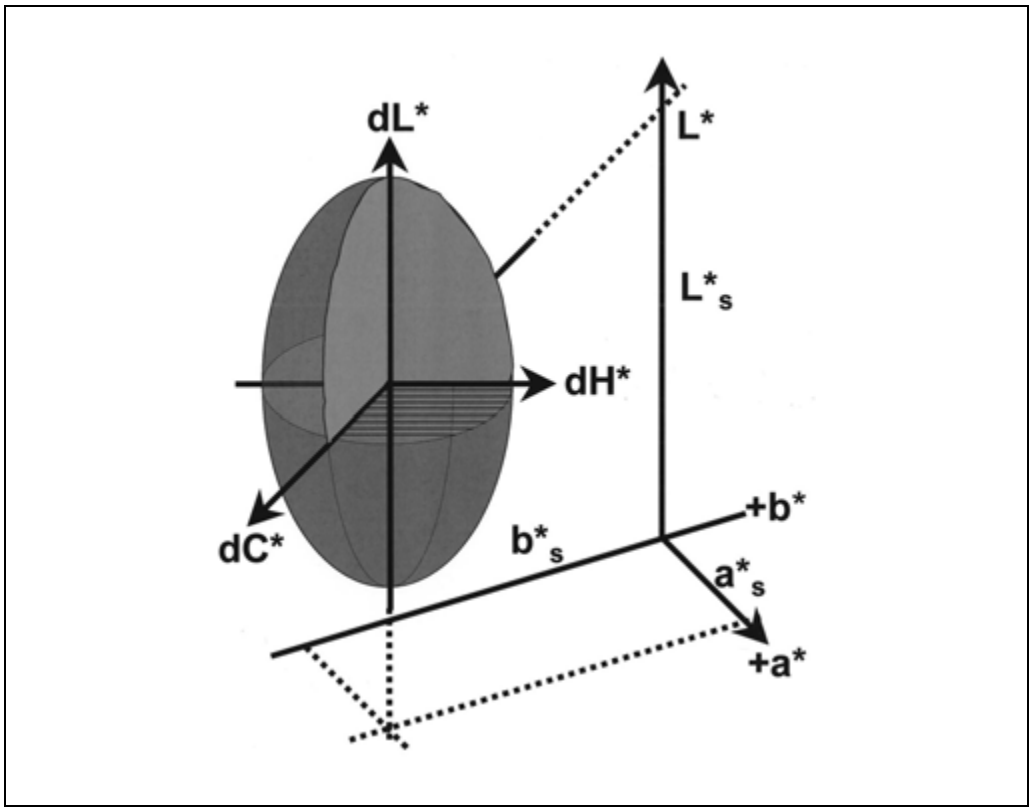

Figure F5.1.15 A representation of a CMC ellipse defines the volume of color acceptability. Figure courtesy of GretagMacbeth. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to http://www.currentprotocols.com/colorfigures*

in many color applications. Some limitations of a calculated *DE** are:

1. It represents a single number that by itself can only indicate the magnitude of color difference, but not the direction.

2. A *DE** = 1.0 does not translate into the same visual difference between all colors.

3. The calculated *DE** may be below the acceptable limit, but the color difference may not be acceptable visually.

There has been a long history of setting color tolerances, and even today this is not always done correctly. The criteria for color acceptability are changing and there are many factors contributing to this change. The world of specifiers and suppliers continues to involve more companies in the color-approval process. A color-control program needs to integrate a basic understanding of the difference between the perceptible limit of color and the acceptable limit. Perceptibility is the point at which a trained eye, under controlled conditions, can just begin to see a color difference.

The perceptibility of observers varies and is not the same for all colors. David MacAdam developed early color data in 1942. The MacAdam ellipses (Fig. F5.1.13) are plotted in the CIE 1931 chromaticity diagram. The ellipse changes in size and shape depending upon the location in color space. These data confirm the variations in color-difference perceptibility throughout color space. Acceptability is the agreed upon limit of color difference from a standard and this depends on many factors, including the buyer and seller.

The emphasis on control in certain color applications has resulted in tighter color tolerances. Color tolerances need to be realistic so they are achievable when considering the variables introduced by people, products, and instruments. The acceptable limits should not be set at the minimum limit of what a human observer can perceive. Added to this is the visual nonuniformity that exists for both perceptible and acceptable limits. Human observers do not respond equally to all colors. Hopefully, a uniform color difference equation could be available that adjusts the perceptibility and acceptability limits for individual colors.

CMC AND BEYOND

Experience has shown that samples evaluated visually, and then measured for color difference, will not all plot within a rectangular-shaped area of acceptability. In actuality, the pattern formed by plotting the samples will be

elliptical in shape. This is why CIELAB rectangular tolerances do not work well when trying to set acceptability limits that agree with the eye (Fig. F5.1.14). In practice, CIELAB accepts some samples that should not be accepted and rejects others that should be accepted.

In 1984, a new Delta *E* (CMC) color-difference formula was recommended for industrial pass/fail decisions. The DE_{CMC} formula defines an acceptability ellipse with the standard located in the center (Fig. F5.1.15). All trials inside the volume of the ellipse are within the CMC tolerance (usually set at $DE_{CMC} = 1.0$) and should be visually acceptable. The DE_{CMC} is calculated from the CIELCH color scale that was published in 1976 along with CIELAB. The CMC ellipse is defined by the *DL*, DC** and *DH** coordinates. The size and shape of the ellipse will vary throughout three-dimensional color space as the standard changes in hue, chroma, and lightness. The hue is considered the most critical dimension in defining the CMC ellipse. The relationship between lightness and chroma is usually set at a 2:1 ratio, with lightness being the least critical dimension. DE_{CMC} has proven it can be applied as a single-number tolerance for all the colors of a given product.

CONCLUSION

Color scientists continue to propose newer color-difference equations. New models of color instruments are being introduced almost every year. The world of color analysis is adapting to the scientific advances that shape food technology and many other areas. Color measurement is still founded on basic principles that confirm the important relationship between the human eye and the instrument. Spectrophotometers and colorimeters assist the eye rather than replace the eye. A properly maintained color instrument is a great asset to a colorist with a trained eye.

The science and methods of colorimetry have been reviewed as a system of color analysis. The color measurement concludes successfully when the instrument data agree with the visual evaluation. The color data from the instrument provide a consistent, objective, and documented way to evaluate color. However, the final color analysis is the judgment made by the eye. Color is neither accepted nor rejected by numbers alone. What really matters in the end is how the object appears.

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A standard work that covers the fundamental principles of color measurement. Included are practical applications of color technology.

Berger-Schunn, A. 1994. Practical Color Measurement, 1st ed. John Wiley & Sons, New York.

Provides details on computerized color measuring systems. Real-life examples are cited along with practical knowledge from the author, who has many years of industry experience.

GretagMacbeth. 1998. Fundamentals of Color and Appearance. GretagMacbeth, South Deerfield, Mass.

A short book designed to provide a basic foundation for understanding color and appearance. It is used in educational seminars intended for people involved in the evaluation and management of color and appearance where fundamental principles can be applied to solve issues.

Hunter, R.S. 1975. The Measurement of Appearance, 1st ed. John Wiley & Sons, New York.

An important textbook and reference that covers color and appearance as it relates to objects and the methods available for measurement.

Contributed by Kevin Loughrey
GretagMacbeth
South Deerfield, Massachusetts

Direct Sampling

Because the chemicals that stimulate the olfactory epithelium are released from the food matrix during eating, a process called retronasal smell, sampling methods for gas chromatography analysis should reflect this release; however, most analyses of aroma chemicals either reflect the composition in the food matrix or the equilibrium gas phase above the food, neither of which truly reflect the composition at the receptor site during eating. An exception is the equilibrium headspace composition, which approximates the composition at the olfactory epithelium when someone sniffs a food orthonasally (Deibler, 1999). This is important when the analyst is concerned about environmental aroma, fragrance materials, or the simple sniffing experience that can accompany eating (e.g., formal wine tasting).

The core technology used in the analysis of aroma chemicals is gas chromatography (GC); therefore, foods must be sampled so they can be introduced on to a GC column. For liquid samples it is possible to inject them into split, splitless, or on-column injectors directly. This is the preferred method for the analysis of synthetic aromas, essential oils, and aroma standards; however, solid or dilute liquid samples need to be extracted, distilled, or gas-phase generated in order to obtain useful results. This unit begins with simple direct analysis of a synthetic flavor (see Basic Protocol 1) followed by the analysis of a dilute liquid sample by solvent extraction (see Basic Protocol 2). It ends with a protocol for determining retention indices (see Support Protocol).

It is highly recommended that all retention properties be normalized to one or preferably several reference standards. Retention indexing, first described by Kováts (Kováts, 1965; see Support Protocol), is by far the most useful standardization procedure for aroma chemicals, because there are published databases (see Internet Resources) listing hundreds of aroma chemicals including their Kováts indices on several substrates.

DIRECT HEADSPACE SAMPLING

BASIC PROTOCOL 1

The simplest and most direct method to analyze for flavor chemicals is to separate them chromatographically and to quantify them with an appropriately sensitive detector. This is generally difficult for two reasons: (1) the chemicals are present at extremely low concentrations, and (2) the sample contains many interfering compounds. For the analyst, it is often a matter of trying a method and then altering it to minimize interferences and enhance sensitivity. Consider a simple example testing the strength of a concentrated Concord grape sample. There are two compounds that are responsible for most of the aroma of Concord grape flavor: methyl anthranilate and β-damascenone. Methyl anthranilate, generally present between 1000 and 10,000 times the concentration of β-damascenone, is the only odor-important volatile that can be detected by direct injection of a headspace sample as described in this protocol. To detect β-damascenone the sample needs to be concentrated; however, the direct extraction described in this unit (see Basic Protocol 2) is still not good enough to give convincing data for β-damascenone using FID detection. More sensitive and selective methods like those in *UNITS G1.2–G1.4* are needed to quantify very odor-active trace components.

Materials

Blank solution (e.g., ~5% ethanol)
Indexing standards solution of *n*-alkanes (C7 to C18 in 0.005% pentane; see recipe)
Essence

Contributed by Terry E. Acree
Current Protocols in Food Analytical Chemistry (2001) G1.1.1-G1.1.10

Gas chromatograph:
Flame ionization detector (FID), 250°C
Column oven
Ov101 or DB5 capillary column (e.g., 20 m × 0.32–mm; f = 0.25 µm; see Table G1.1.1)
Helium linear velocity: 36 cm/sec (~2 ml/min)
Injector, 250°C
10-ml gas-tight syringe
100-ml flask fitted with a rubber septum

1. Program the GC oven to run isothermal for 3 min and then program to increase 2° to 6°C/min to 225°C. Hold for 10 min.
2. Inject 1 µl blank solution (e.g., ~5% ethanol) into the GC and start the program.

   This will show any artifacts or contaminants from either the injector or the column.
3. Inject 1 µl indexing solution of *n*-alkanes and start the GC program.
4. Record the retention time for each *n*-alkane to be used for indexing (see Support Protocol).
5. Place 50 ml essence in a 100-ml flask fitted with a rubber septum and allow it to equilibrate for an hour or more.
6. Withdraw 5 ml headspace gas from above the essence sample into a 10-ml gas-tight syringe. Inject slowly into the GC.
7. Record the retention time of each peak that elutes.

   Use the data from the n-alkanes to convert each time into a retention index (see Support Protocol).
8. Use a data base of retention indices (e.g., the Flavornet; see Internet Resources) to tentatively identify each peak.
9. Quantify peaks of interest (e.g., methyl anthranilate in Concord grape samples).

Table G1.1.1 GC Substrates, Polarity, and Other Phases of Similar Polarity

Phase	Polarity	Phases of similar polarity
OV-101	Nonpolar	OV-1, SP-2100, Apiezon L., DC11, DC-200, JXR Silicone, UC-W98X, SPB-1, SE-30, BP-1, CP Sil5CB, DB-1, DC-200, GB-1, OV-1CB, OV101
DB-5	Very slightly polar	SPB-5, SE-54, PTE-5, BP-5, CPSil 8CB, GB-5, 007-2, PVMS-54, Rtx-5, SE-52, SE-52CB, SE-54CB, RSL-200, Ultra-2
OV-17	Intermediate	SBP-20, SPB-35, SPB-1701, DB-1301, DC-550, Dexsil 400, 007-7, 00-17, OV-17, OV-1701, Rtx-20, SP-2250, DB17, GB-17, HP-17, PVMS-17, RSL-17, Rtx-50
C20M	Polar	Nukol, SP-1000, AT-1000, BP-20, CP-Wax51, FFAP, FFAP-CB, H-FFAP, OV-351, Stabilwax-DA, Superox, BP-20, CP Wax57CB, DB Wax, Pluronics L-121, RSL-310, SUPELCOWAX 10, Stabilwax, Superox 20M
OV-275	Very polar	SP-2330, SP-2331, SP-2380, CP SIL 84, Rtx-2330, SH-60, SH-70, Silar 9 CP, CPS1, CPS2, CP SIL 88, Rtx-2330, Rtx-2340, SH-80, SH-90, Silar 10CP

SOLVENT EXTRACTION

The most common method for the separation and concentration of flavor chemicals before chromatography is solvent extraction. If the aroma active components in a sample are less than a microgram/liter then solvent extraction followed by fractional distillation can be used to concentrate the analytes above 1 µg/liter. This is done for two reasons: (1) to remove the odorants from some of the interfering substances and nonvolatiles, and (2) to concentrate the sample for greater sensitivity. The choice of solvent(s) depends on a number of issues, but similar results can be obtained with many solvents. Table G1.1.2 lists a number of solvents, their polarity, and physical properties. Pentane is the least polar and ethyl acetate the most. The sample must be an aqueous or dilute sample, dissolved or slurried into water to a final concentration of 80% to 90% water. Dilute aqueous samples will present the greatest polarity difference between the solvent and the sample, driving more volatiles into the extracting solvent.

Materials

Sample
Chromatographic grade pentane
$MgSO_4$, anhydrous
Ethyl acetate (optional)

2-liter extraction flask (fermentation or Erlenmeyer flask)
2-liter separatory funnel with Teflon stopcock and ring stand
Liquid and powder funnels
250-ml rotary evaporator flask
Rotary evaporator:
  Pressure gauge
  Valve for a vacuum break
  Needle valve to control the pressure
  Waterbath to maintain temperature between 10° and 30°C
  10-ml pear-shaped rotary evaporator flask

1. Place 1 liter sample and a Teflon- or glass-coated stirring bar in a 2-liter extraction flask.
2. Gently (to prevent emulsions) add 600 ml chromatographic grade pentane.

   The amount of solvent should be more than half but less than the total sample volume. All analysis should be done at the same sample to solvent ratio (e.g., 0.6:1.0).

Table G1.1.2 Polarity, Boiling Points, and Flash Points of Solvents Used for Extraction of Volatiles from Foods

Name	Polarity	Boiling Point (°C)	Flash point (°C)
n-pentane	0.00	35–37	−40
n-hexane	0.00	68–69	−22
carbon disulfide	0.15	46.3	−30
carbon tetrachloride	0.18	76–77	NA[a]
ethyl ether	0.38	34–35	−45
methylene chloride	0.42	40–41	NA[a]
ethyl acetate	0.58	77–78	+7.2
ethanol[b]	0.88	78.5	+13
methanol[b]	0.95	64–65	+12
water[b]	large	100	NA[a]

[a]NA is not available.
[b]These solvents are used to extract volatiles from solids followed by extraction with one of the other solvents.

3. Place the extraction flask on a magnetic stirrer and adjust the speed to <1 rotation per sec (~30 rpm) for 20 min.

   NOTE: *Do not let a cyclonic vortex develop between the solvent and sample layers. This can cause an emulsion to form. A slight dent should be visible in the surface of the solvent layer as the stirring bar sets up a toroidal current in the sample layer and this is transferred to the solvent layer.*

   Unlike many of the common extraction protocols used in organic chemistry the extraction of odorants from complex foods will result in seemingly unbreakable emulsions because of the fats and proteins they often contain; therefore, shaking the phases is seldom recommended.

4. Set up a 2-liter separatory funnel on a ring stand as shown in Figure G1.1.1. Use a liquid funnel to help direct the liquids into the separatory funnel, and a powder funnel with shark skin–fluted filter paper containing 50 g anhydrous magnesium sulfate on a 1-liter receiver flask.

   If the bottom phase is the aqueous phase use a receiver flask without magnesium sulfate first.

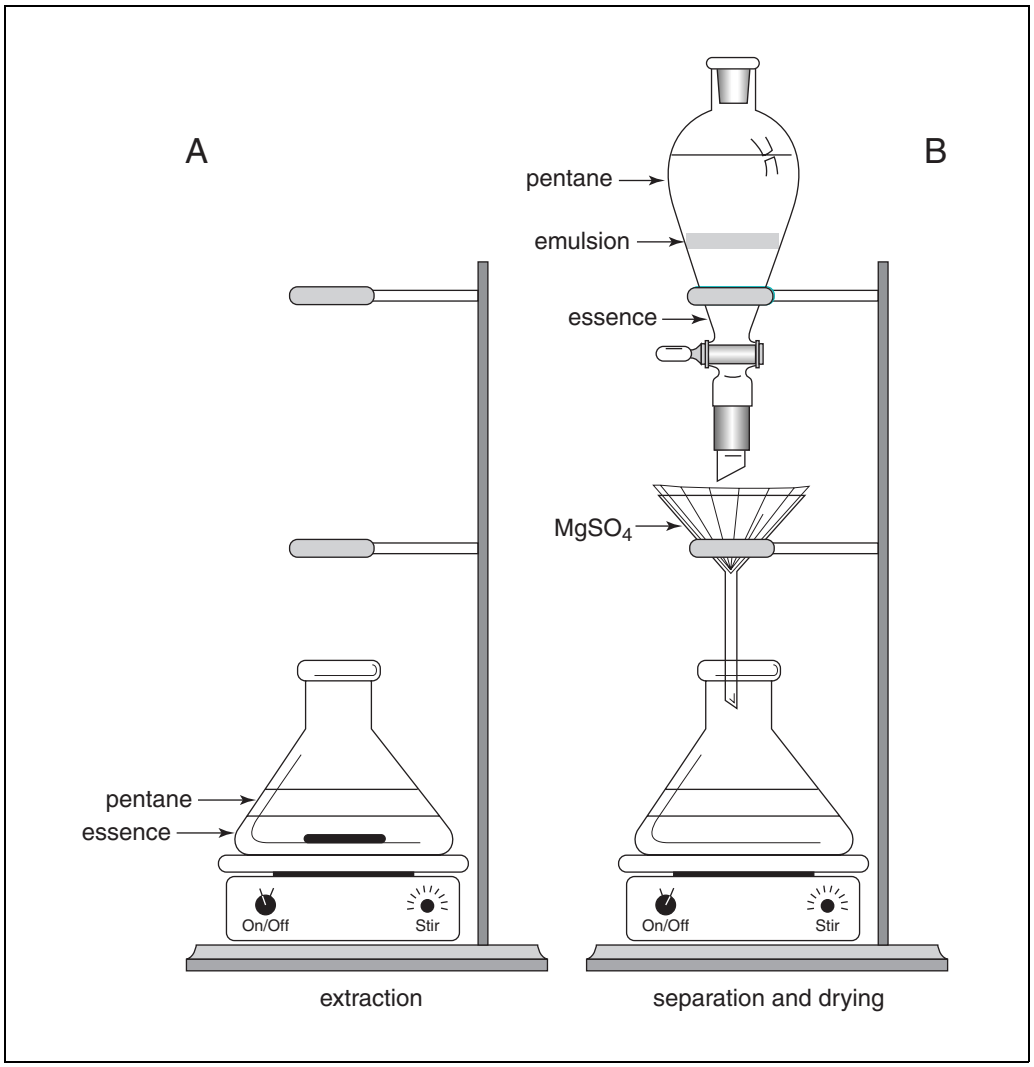

Figure G1.1.1 The apparatus used to extract liquid samples. The size of the glassware will depend on the scale of the samples. For example, a 500 ml sample will require a 2-liter fermentation flask for extraction but a 1-liter separatory funnel.

5. Remove the magnetic stirring bar and carefully pour the contents of the extraction flask into the separatory funnel. Allow the layers to separate.

   A rod can be used to direct liquids into the separatory funnel with a minimum of emulsion formation.

6. Allow the phases to separate (this may take as long as 1 or 2 hr).

   The goal is to have at least 90% of the organic phase free from any emulsion.

7. Separate the phases carefully, leaving any emulsion in the aqueous phase. Since pentane is less dense than water, allow the water phase and emulsions to drain directly into a 1-liter Erlenmeyer flask. If the odorant is highly polar, save the aqueous phase for extraction with a more polar solvent in step 9, otherwise discard.

8. Allow the organic phase to drain as quickly as possible through the magnesium sulfate.

   Don't let it overflow the powder layer and run directly through the filter paper and into the funnel. This is the nonpolar extract.

For highly polar odorants:

9. (Optional) If the sample contains highly polar odorants, set up the extraction system again and put the extracted aqueous phase back into the extraction flask. Add 600 ml ethyl acetate and repeat steps 2 through 6. This is the polar extract.

   This step is used to extract the sample with a more polar solvent.

   Uncleaned glassware from the nonpolar extraction can be used in this step.

Concentrate the sample

10. Concentrate the organic phase to ~30 ml by placing ~150 ml at a time into a 250-ml rotary evaporator flask. Place the flask in the rotary evaporator, turn on the vacuum, and close the vacuum break. Using the needle valve, allow the vacuum to increase until solvent begins to condense. Add another aliquot of the extract (150 ml total) until all the extract has been concentrated to ~30 ml.

    NOTE: *Do not distill the solvent at too-low pressure (i.e., high vacuum) or the highly volatile odorants will be lost.*

    Place concentrate in brown glass bottles with Teflon lined caps. The sample can be stored at this concentration for many months. Room temperature is fine but refrigerators often have a more constant temperature than laboratories.

11. Concentrate the sample down to 1 ml in a 10-ml pear-shaped rotary evaporator flask. Run as described (see Basic Protocol 1).

    NOTE: *When using solvents that absorb a lot of water (e.g., ethyl acetate, diethyl ether) it is necessary to dry the extract a second time with anhydrous magnesium sulfate (1 g/10 ml sample). This gives samples that are ready for gas chromatography that are 1000× more concentrated than the original sample.*

RETENTION INDEXING

SUPPORT PROTOCOL

The usefulness of retention data from gas chromatography can be enhanced by reporting standardized times or retention indices (RI), which involves expressing retention in terms of a ratio of the retention time (RT) of an analyte to the RT of a standard. Retention scaling based on the Kováts (1965) method requires the chromatographic separation of a homologous series of normal paraffins, esters, and others, producing an index that is the ratio of the RT of an analyte minus the RT of a less retentive standard to the RT difference between

a less retentive standard and the next most retentive standard (Figure G1.1.2). Retention times are so dependent on experimental conditions that it should be a rule of analytical chemistry that all retention times be reported as standardized indices. For the last twenty years the most useful resource in laboratories using gas chromatography/olfactometry (GC/O) has been a compilation of retention indices for flavor compounds (Jennings and Shibamoto, 1980). More recently, databases published on the world wide web have become even more useful (see Internet Resources).

See Basic Protocol 1 for materials.

1. Inject 1 µl standard solution into the GC under the same conditions used to chromatograph the sample. Start the program, and determine the retention time for all paraffin standards (see Basic Protocol 1).

   For example, programming the methyl silicone OV-101 (or DB-1, SE-30, or equivalent) column to run isothermally at 35°C for 3 min, then increasing oven temperature 4°C/min to 225°C, and finally holding the temperature isothermal for 10 min, will elute all the paraffins from C7 to C18 in 30 min. These are good conditions for the analysis of fruit-juice essences.

   The exact chromatographic conditions must be determined from the properties of the samples being analyzed and the goal of the analysis. The same conditions and column must be used to run the indexing standards. Once the chromatographic conditions have been chosen, it is a good idea to test them by running the indexing standards first. The chromatogram of the standards can establish sensitivity of the detector, the performance of the column and the chromatograph before the sample is tested.

2. Calculate the retention index of all the peaks of interest in the chromatogram using the following formula.

   $RI_x = [(T_x - T_n)/(T_{n+1} - T_n)] \times (n \times 100)$

   Where:

   T_x = retention time of a peak (x) to be indexed

   T_n = retention time of the paraffin that immediately precedes x

   T_{n+1} = retention time of the paraffin that immediately follows x

   RI_x = Kováts retention index of x

   n = paraffin carbon number of the paraffin that immediately precedes x

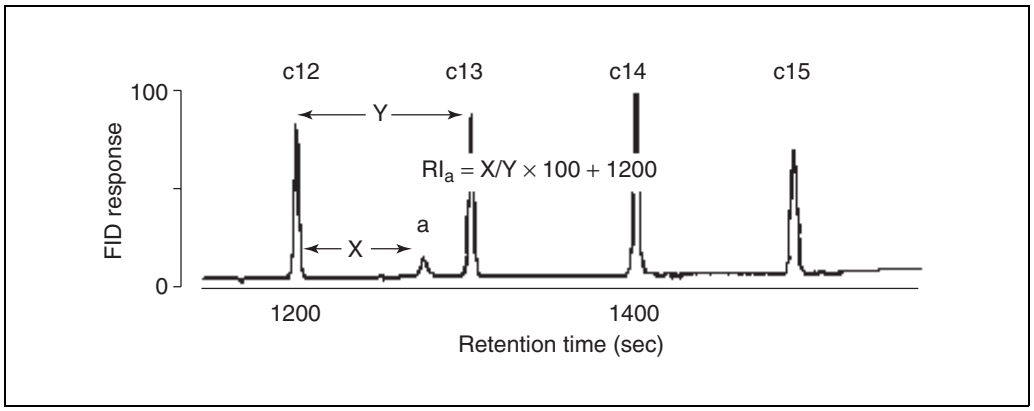

Figure G1.1.2 An FID chromatogram of an indexing standard and parameters used to calculate retention indices. For equations, see Support Protocol, step 3.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Paraffin stock solution, 1000 ppm

Using a micropipet, add one drop of each *n*-paraffin from heptane to hexadecane into a 100-ml brown glass bottle containing 50 ml of an appropriate solvent (e.g., pentane for nonpolar columns). Seal with a Teflon-lined screw cap. Store at 4°C in the refrigerator for years.

Adding 2 drops of one of the paraffins in the middle (e.g., tridecane) will yield a chromatographic pattern that makes it easy to identify all the peaks quickly. Knowledge of the exact concentration of the paraffins is not essential for indexing, but such information could be used to monitor GC performance.

Indexing standards solution, 10 ng/µl

Dilute an aliquot of the paraffin stock solution 1:100 to produce a 10 ng/µl solution. For example, add 5 ml solvent followed by 50 µl paraffin stock solution (see recipe) to a 10-ml brown bottle and seal with a Teflon-lined screw cap. Store at room temperature near the GC for months if necessary.

COMMENTARY

Background Information

Any number of extraction protocols can be used to prepare samples for gas chromatography, but in every case the goal is to simplify the chromatogram and amplify the response for the odorant. The protocol given here covers the broadest range of polarities possible using solvent extraction from an essentially aqueous sample. This and similar extraction methods are ideal for many fruit and vegetable products, homogenized meat products, and grain products ground into a slurry with water. There have been many papers comparing the virtues of different solvent extraction protocols (Leahy and Reineccius, 1984) and all have some limitation. In the protocol reported here, the use of pentane and ethyl acetate will provide an excellent result with a wide range of samples. In addition, except for their fire and explosive potentials, they are very safe solvents to use, as they have a low toxicity and no potential to accumulate in the body.

There are many samples that can be injected into a gas chromatograph in order to determine what aroma active volatile is present or to quantify a particular one (Maarse and van der Heij, 1994). Unfortunately, many of the protocols that were followed in the past did not rely on meaningful standards or retention indexing, or failed to use GC sniffing or the more formal GC/O procedures to establish that the peaks detected had odor activity; however, if the object of the analysis is to simply monitor one or two odorants known to contribute positively or negatively to the aroma of a sample, the procedure should be optimized to analyze the specific odorants accurately, precisely, and efficiently.

Critical Parameters and Troubleshooting

To do direct injections (see Basic Protocol 1) requires that the sample be low in nonvolatiles (e.g., fats, proteins, and carbohydrates). The column properties should be appropriate to achieve the objective of the analysis. For example, the ability to resolve methyl anthranilate from other analytes is critical for the assessment of flavor quality in Concord grape, but in the analysis for geosmin contamination of drinking water, sensitivity to chemicals at less than a nanogram per liter is essential. In this case a concentration step is necessary. When a sample is in a solvent that interferes with the vaporization process—e.g., ethanol, polypropylene glycol (a common carrier for synthetic flavors), and others—improved results (i.e., less peak broadening at the start of the chromatogram) can be obtained by simply adding an equal amount of a highly volatile solvent (low heat of vaporization) such as diethyl ether, preferably redistilled. This will aid in the transfer of the sample to the column and the extraction of the analytes by the substrate, and is often necessary for good splitless or on-column injections. Usually, limiting the concentration of any highly polar solvents (e.g., ethanol, polyethylene glycol) to <25% will minimize this problem.

Most if not all aroma compounds have a molecular weight of less than 350 amu and can be chromatographed within the operating temperatures of many GC substrates, especially the methyl silicones. Although the choice of substrate depends on the goals of the analysis, most flavor chemists use nonpolar substrates (e.g., SE-30, SE-54, OV-101) for samples low in polar components, and polar substrates for samples in which polar compounds (e.g., volatile acids) have a significant effect on the analysis. As pointed out in *UNIT G1.3*, the use of both substrates are required when GC retention properties are being used for identification purposes; however, it is always a good idea to use substrates that are common in the literature in order to produce data that is comparable with published data. For example, the "Flavornet" and the "Threshold" data bases on the world wide web present data for aroma compounds separated on OV-101, DB-5, OV-17, C20M, or their equivalents (see Internet Resources). These are good substrates to consider first. Table G1.1.1 list these substrates and their equivalents among the many commercially available GC stationary phases.

The types of general detectors most commonly used for the analysis of aroma compounds include flame ionization detection (FID) and electron impact mass spectroscopy (EI/MS). Although a score of special detectors can be used to detect specific aroma compounds, the three most commonly used are mass fragmentography, often called selected ion monitoring (SIM, a registered trademark of Agilent Technologies), chemical ionization mass spectroscopy (CI/MS), and GC/O. The single most commonly used among these is GC/O, since it can yield odor activity measurements directly (Acree, 1997). Furthermore,

Table G1.1.3 Troubleshooting Table

Problem	Possible cause	Solution
Compounds do not match the reported index	A damaged or contaminated column	Use a new or different column (DB-5 is the most stable column for both polar and nonpolar compounds)
Compounds do not match the index of the same standard in a different solvent	Solvent effect on the elution of the standard	Use standards dissolved in the same solvent the analytes will be dissolved in

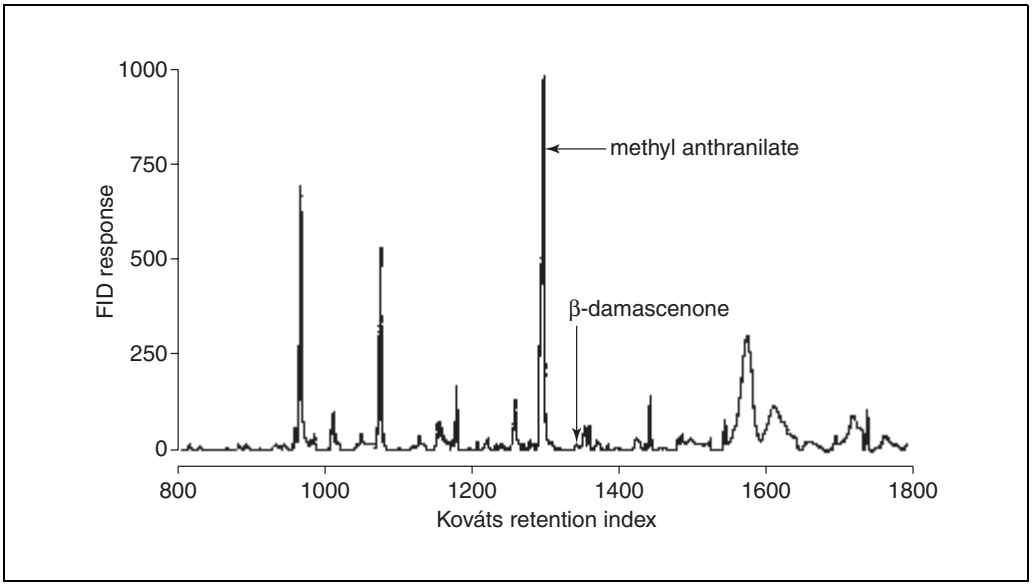

Figure G1.1.3 FID gas chromatogram of a direct injection of the headspace above concentrated extract of Concord grape essence using OV 101 substrate. Note the size of the methyl anthranilate peak and the absence of a convincing peak for β-damascenone.

when GC/O is combined with the use of authentic standards, it is the most robust method for the identification of odorants.

Solvent extraction

A number of solvents have been used to extract volatiles for aroma analysis but the optimum choice depends on a compromise. Table G1.1.2 lists the most common solvents used to extract odorants from foods. Although pentane and ethyl acetate are flammable, they have a very low toxicity, represent extremes in polarity, and a sequential extraction using these two solvents will remove most of the volatile odorants from aqueous samples (see Basic Protocol 2); however, if the desire is to do a simpler one-step extraction, then a solvent should be chosen with a polarity that will extract the volatiles of interest. For example, maltol is not extracted well with pentane, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone, the smell of strawberry, is almost insoluble; therefore, the choice of the optimum solvent depends on the analyte and may require some testing to find.

Retention indexing

This method (see Support Protocol) requires a set of *n*-paraffins or *n*-ethyl esters to produce data that is comparable to that published on the internet. Table G1.1.3, the troubleshooting table below, lists some of the frequent problems encountered, their probable cause and possible solution.

Anticipated Results

Figure G1.1.3 shows a chromatogram of the headspace of Concord grape essence prepared by direct injection. At retention index 1320 is the peak caused by methyl anthranilate, one of the strongest odorants characterizing Concord grapes; however, β-damascenone, the second most potent odorant in Concord grapes, elutes at 1360 but is not visible. This is because β-damascenone is 1000× more potent (i.e., its odor threshold is 1000× lower than methyl anthranilate). This is typical result for the direct injection of headspace from natural products. Figure G1.1.4, on the other hand, shows the injection of an extract of Concord grape essence concentrated 500-fold with the β-damascenone peak large enough for quantitation.

Time Considerations

It takes ~1 hr to run a blank, and another hour to run the alkane standards by direct headspace sampling (see Basic Protocol 1). With OV-101 and similar substrates, these standards need be run only once every day or two. With more polar and less stable substrates, or with samples that contain a lot of nonvolatile material, the standards and blanks need to be run more often. In the worst case, these must be run

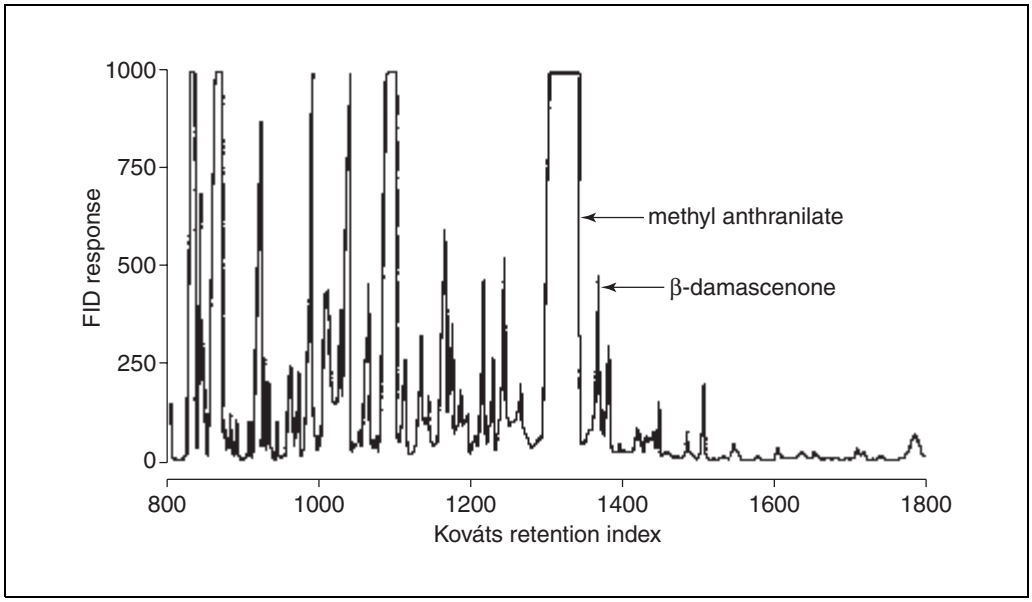

Figure G1.1.4 An FID chromatogram of concentrated extract of the same Concord grape essence shown Figure G1.1.3, drawn to display the data on a linear retention index scale. By simply comparing the index of a peak with the data listed in the flavornet, the odorants that have similar retention indices can be determined. Notice how large the methyl anthranilate peak is, but still no convincing peak for β-damascenone, even though both compounds have the same odor activity (intensity).

before and after each sample. Under these conditions, only a couple of samples can be run in a day. In the best case it should take ~1 hr to run a sample including the recycle time for the GC oven.

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Maarse, H. and van der Heij, D.G. 1994. Trends in flavour research. *Dev. Food Sci.* 35:516.

Key References

Acree, T.E. and Teranishi, R. 1993. Flavor Science: Sensible Principles and Techniques. American Chemical Society, Washington DC.

A monograph that touches upon most of the important issues in flavor clinical analysis.

Belitz, H.-D. and Grosch, W. 1999. Food Chemistry: Second Edition. Springer, Berlin.

Textbook on food chemistry with an excellent discussion of flavor chemicals.

Internet Resources

http://www.nysaes.cornell.edu/flavornet

Contains retention indicies on 4 substrates, CAS numbers, and odor qualities for over 400 chemicals identified by GC/O in food.

http://www.odor-thresholds.de

Contains thresholds and retention indices for several 100 odorants.

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Isolation and Concentration of Aroma Compounds

Aroma compounds are present in minute levels in foods, often at the ppb level (µg/liter). In order to analyze compounds at these levels, isolation and concentration techniques are needed. However, isolation of aroma compounds from a food matrix, which contains proteins, fats, and carbohydrates, is not always simple. For foods without fat, solvent extraction (*UNIT G1.1*) can be used. In foods containing fat, simultaneous distillation extraction (SDE; see Basic Protocol 1) provides an excellent option. Concentration of headspace gases onto volatile traps allows sampling of the headspace in order to obtain sufficient material for identification of more volatile compounds. A separate protocol (see Basic Protocol 2) shows how volatile traps can be used and then desorbed thermally directly onto a GC column. For both protocols, the subsequent separation by GC and identification by appropriate detectors is described in *UNIT G1.3.*

SIMULTANEOUS DISTILLATION EXTRACTION

BASIC PROTOCOL 1

One design of the micro steam simultaneous distillation extraction apparatus is given in Figure G1.2.1. Both sample and solvent flasks are heated to their boiling points. Vapors coming from the sample and solvent flasks (Figure G1.2.1, G and F) are mixed in the

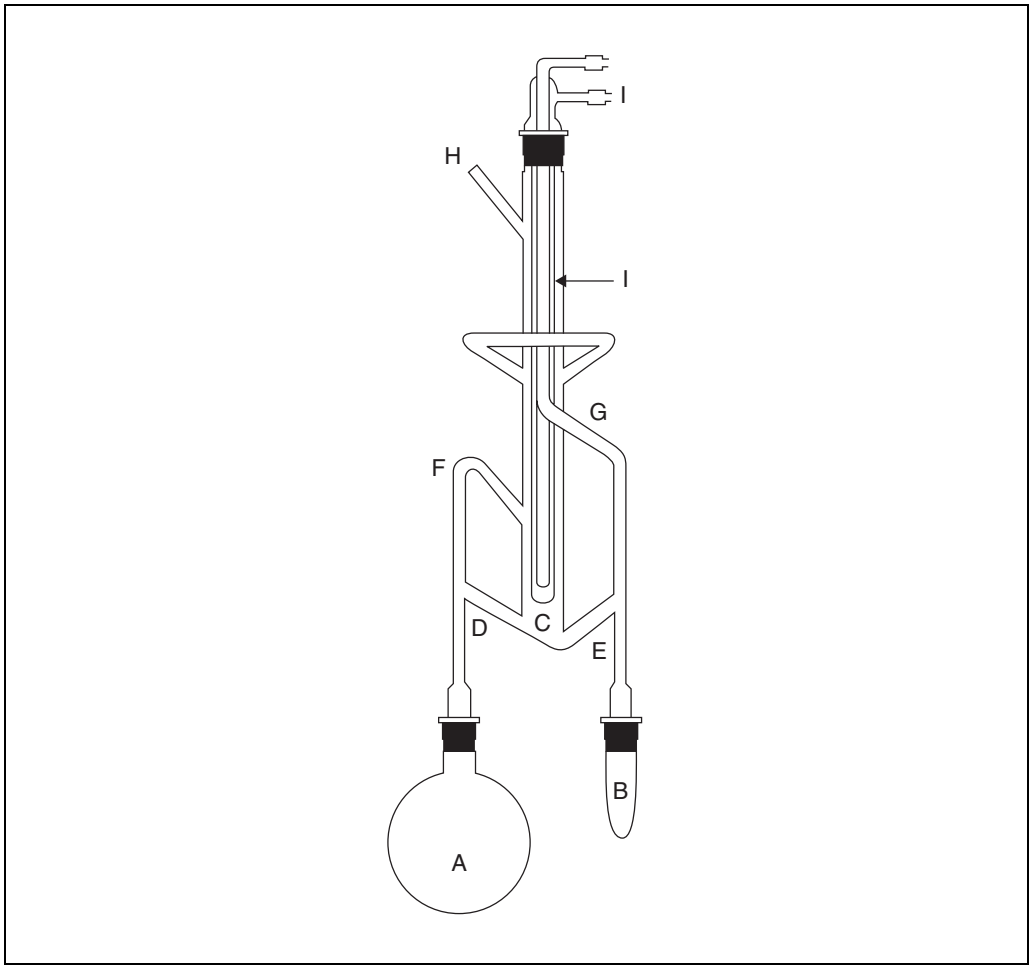

Figure G1.2.1 Simultaneous distillation extraction system. A, flask for water; B, flask for solvent; C, separation chamber; D, return tube for water; E, return tube for solvent; F, vapor tube for water; G, vapor tube for solvent; H, inlet/vent; I, cold finger. Modified from Godefroot et al. (1981).

Contributed by Deborah Roberts and Hugues Brevard
Current Protocols in Food Analytical Chemistry (2001) G1.2.1-G1.2.9

central part of the apparatus (Figure G1.2.1, C) and condensed on the cold finger (Figure G1.2.1, I) . Volatiles are transferred from the water phase to the organic solvent. The organic and water phases return to their original flasks through Figure G1.2.1, D and E tubular parts. Gradually the volatile compounds are transferred from the sample to the organic solvent. At any time during the extraction, there is no contact between the solvent and the matrix.

Materials

Deionized water
Sample
Sodium chloride (optional)
Internal standard (optional)
Buffer (optional)
Organic solvent, freshly distilled or with high purity (see Table G.1.2.1)
Drying agent (e.g., magnesium or sodium sulfate)

Simultaneous distillation extractor, which may vary in the design with respect to the solvent density (e.g., Chrompack, Alltech, or many glass-blowers)
Cooling bath
Heating bath
Stirring plate

1. Degas deionized water by vacuum, while stirring for 10 min.

   Optional: Saturate with sodium chloride, add an internal standard (e.g., 250 µl of solution containing 25 µg of internal standard), and add buffer.

   Degassing allows the elimination of residual oxygen that may modify the nature of certain chemical compounds. A better aroma recovery is obtained when the aqueous phase is saturated with salt, while an internal standard addition allows semi-quantification.

2. Cool the cold finger condenser of the simultaneous distillation extractor to 0°C, using a cooling bath.

3a. *For analytic purposes:* In the <500-ml sample flask (see Fig. G1.2.1A) containing a stir bar, place a small quantity of sample, i.e., <20 g with ~250 ml of water.

   Alternatively, an internal standard, sodium chloride, and buffer can be added in place of water.

Table G1.2.1 Commonly Used Solvents

Example of solvents	Boiling point (°C)
Diethyl ether	34.6
Pentane	35
Dichloromethane	40
Chloroform	61
Hexane	69
Trichloroethylene	87
Heptane	99
Isooctane	99
2-Pentanone	102
Trichloroethane	110
Toluene	111
Octane	125

3b. *For preparative purposes:* In 2- to 3-liter sample flask containing a stir bar, place a higher quantity of sample, i.e., 20 to 1000 g with ~250 ml of water.

4. Place adequate quantities of organic solvent (see Table G1.2.1) in solvent flask (see Fig. G1.2.1B) containing a stir bar.

   Typical quantities vary between 4 and 5 g of solvent for analytical purposes and between 40 to 50 g for preparative purposes.

   The solvent must be nonmiscible with water and should have a boiling point as low as possible. Solvents such as dichloromethane (higher density than water) and diethyl ether, pentane, or the mixture of both (lower density than water) are commonly used. Freons, chlorinated solvents, or alkanes can be used. Toxic solvent should be avoided.

5a. *For a solvent heavier than water:* Start heating the solvent first in order to fill the central part of the extractor (see Fig. G1.2.1 C) full of solvent, then start to distill the water. Heat to the boiling point of the liquids and continue the distillations.

5b. *For a a solvent with a lower density than water:* Start heating the water flask first. Heat to the boiling point of the liquids and continue the distillations.

   If sample does not contain fat or oil, the duration of the extraction should not exceed 1 hr. If it contains fats one must verify the extraction time that is necessary for an efficient aroma recovery. Depending on the matrix, one must also verify the appropriate extraction time, prior to beginning. Generally, this time does not exceed 1 hr. Solvent injection and quantification by GC/MS after the SDE is usually used to verify the minimum extraction time needed.

6. Remove the heating plate or bath from the solvent flask and let the distillation of the sample continue for 10 min.

   This will allow a better recovery of volatiles contained in tubular parts Figure G1.2.1, D and E.

7. Collect the organic phase and add a drying agent, typically magnesium or sodium sulfate.

   For 5 g of solvent, ~100 mg of drying agent is sufficient.

8. Inject the organic phase without or after concentration onto a GC column in splitless or on-column mode.

   Concentration can be performed under a gentle stream of inert gas or with a micro-concentration apparatus (e.g., Kuderna-Danish sample concentrator, Supelco, or microconcentrator). This step generates volatile losses (mainly very volatile compounds that have a boiling point lower than the solvent) and will modify the quantitative ratio.

VOLATILE TRAPS FOR CONCENTRATION: ADSORPTION AND DESORPTION

BASIC PROTOCOL 2

Adsorbent traps can be used to concentrate volatile compounds without solvents so that the minute quantities present in a product (often ppb levels for odor-impact compounds) can be detected. Three steps are involved: (1) concentration of headspace gas on a trap, (2) thermal desorption of the trap and transfer of volatiles to a gas chromatograph, and (3) cryofocusing of the volatiles at the beginning of the GC column. The subsequent separation by GC and identification by an appropriate detector is the subject of *UNIT G1.3*. Several of the specifics of this methodology are related to the particular thermal desorber, which may use different sizes and types of traps and have different construction designs. Although several on-line systems are available that incorporate all three steps, the methodology here will describe off-line systems.

Table G1.2.2 Several of the Most Popular Adsorbents for Volatile Traps

Absorbent type	Advantages	Limitations	Typical use
Tenax TA 60-80 mesh	Good thermal stability and desorption. Traps little water. Generates few artifacts.	Limited adsorption and breakthrough of very volatile compounds	For general volatile trapping. Among the most used adsorbents
Carbon molecular sieves, e.g., Carbosieves, Carboxen, and Ambersorb	Traps small volatiles well	Other volatiles not well trapped	In combination with other adsorbents
Activated charcoal	High retention of nonpolar volatiles	Water absorption. Artifact formation. Some difficulties with desorption	Less used for aroma compounds
Graphitized carbon (e.g., Carbotrap, Carbopack)	Trap hydrophobic compounds. Low affinity for water	Generally used with another adsorbent	Preferably chosen over activated charcoal for general volatile trapping

Materials

5% (v/v) dimethyldichlorosilane in toluene
Adsorbent material (see Critical Parameters and Table G1.2.2)

Silane-treated glass wool (Supelco)
Ultrasonic bath containing 1:1 (v/v) dichloromethane/methanol
100°C oven
Trap (e.g., see Figure G1.2.2) and trap caps
Vessel with connections for "purge-and-trap" (e.g., see Figure G1.2.2)
Thermal desorption system with cold trap
Gas chromatograph with detector

Prepare traps

1. Place small amount of silane-treated glass wool in trap (label this side of trap).
2. Silanize interior with 5% dimethyldichlorosilane in toluene for 5 min, rinse two times, 30 min each, in an ultrasonic bath containing 1:1 dichloromethane/methanol solution, then dry ≥2 hr in a 100°C oven.
3. Fill trap with ~150 to 250 mg adsorbent material.

   See Critical Parameters for a discussion of various adsorbent material options.
4. Place another glass wool plug at the top of the trap.
5. Precondition the traps by heating in the thermal desorber at the time recommended.

   For Tenax, 300°C for 2 hr is recommended. Thermal desorption of traps should be done with labeled side at the gas exit.
6. Keep the traps stored with their caps on in an odor-free environment such as a dessicator with positive nitrogen pressure.

   If the traps have not been used in a week, recondition them by heating for 10 min at the temperature recommended.

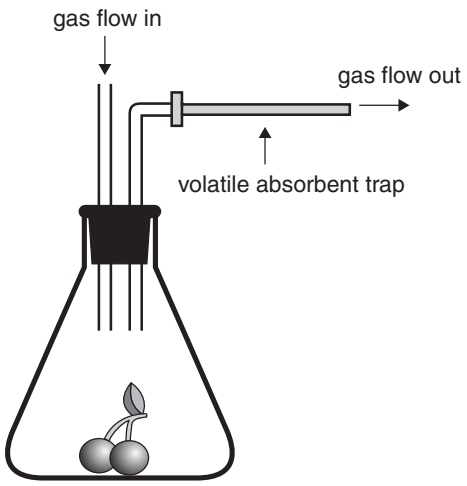

Figure G1.2.2 Typical set-up for dynamic headspace sampling using a volatile adsorbent trap.

Trap aroma compounds

A number of different set-ups can be used. Figure G1.2.2 shows one possible set-up of dynamic headspace trapping. Normally, the vessel contains the food product, an inlet for nitrogen gas, and an outlet connected to the trap. The inlet could be directed through the liquid food, often called "purge-and-trap," or above the food, often called dynamic headspace sampling.

7. Determine the time and nitrogen flow rate so that breakthrough (no more retention for a given substance) of the trap does not occur. Do this by attaching a second trap in series and analyzing this trap. For aroma compounds, smell the end of the trap to detect odor-active compound leakage. Even if the vessel is heated, the trap should be kept at room temperature.

   Trapping should be done with the labeled side at the gas entry.

8. Remove the trap and place caps on the two ends.

9. Analyze using thermal desorption system after optimization of conditions.

Desorb traps thermally and trap cryogenically

10. Place the trap in the desorber with the labeled side toward the gas exit.

11. Determine conditions of optimal desorption of traps: flow rate, temperature of traps, and transfer lines.

12. Test with a mixture of compounds of different retention indices by injecting 1 µl of compound dissolved in solvent at ~10 ppm levels onto the glass wool at the labeled side.

    For Tenax traps, a 250°C desorption temperature is recommended.

13. Adjust the cold trap to as cold a temperature as possible (temperatures between −100°C and −140°C are commonly used), and adapt as a function of the volatile compounds.

    Cryogenic trapping is used to ensure that all compounds that are desorbed from the trap begin gas chromatography at the same instant. While other types of oven cooling, for

example with carbon dioxide, may retard the migration of compounds on the column, the chromatographic resolution will not be as good as with cryogenic trapping. Depending on the commercial thermal desorption system used, cryogenic trapping may be part of the instrument, or at the beginning of the column. Placing the first loop of the GC column in a Dewar of liquid nitrogen is also valid although the Dewar must be removed at the beginning of the GC run.

Cold trap heating should be done as quickly as possible for injection-like resolution.

GC analysis followed by detection as explained further in UNIT G1.3.

14. Bake the adsorbent trap at an elevated temperature with flow in the reverse direction so it is free from volatiles for the next analysis.

    Run blank trap(s) between series to ensure no carry-over contamination peaks. Also, for each sample type, check the trap to ensure that all volatiles have been desorbed during the bake-out.

15. Do a desorption of alkane standards added to the trap as in step 1 in order to establish retention indices.

    This is done in a separate experiment but in the same way as the sample analysis.

    Alkane standards can be prepared by dissolving 1 mg/liter of C5-C20 alkanes in water, and then injecting 5 μl on the trap.

COMMENTARY

Background Information

Of the two techniques described here, simultaneous distillation extraction (SDE) is a more complete volatile extraction procedure that serves to obtain quantitative information on the compounds contained in a food. Volatile trapping is a partial extraction procedure that samples the volatiles present in the headspace above a food, which are those with higher volatility. Extended trapping also induces additional volatilization of compounds initially contained in the food.

SDE

The main advantage of SDE over direct solvent extraction is the absence of contact between the matrix and the organic solvent during the extraction procedure. This allows foods of various compositions, including fat, to be analyzed. Generally, compounds that are not soluble in water are well extracted. The major drawback of such an SDE is that very soluble compounds generally stay in the water phase and are not efficiently extracted (e.g., vanillin, furaneol, phenylethylalcohol). Another drawback is the possible formation of thermal artifacts. This will not occur with thermally treated samples (i.e., coffee, cooked meat, cocoa) but will happen with fresh vegetables such as garlic and onion. Furfural, which is mentioned in many foodstuffs, is the major thermal artifact.

Different apparatus designs were proposed by several authors (Likens and Nickerson, 1964; Schultz et al., 1977; Godefroot et al., 1981). Maignial et al. (1992) developed a system for isolation of volatile compounds at room temperature by using a closed system under static vacuum. Later, Pollien and Chaintreau (1997) built a preparative device for up to 5 to 10 kg of material which is a semi-static system able to work under vacuum. Working under vacuum reduces thermal artifacts, which may occur with SDE experimentation. Samples in this case are steam distilled at ambient temperature. A solvent with a higher boiling point than that found in conventional SDE is used, which may cover up target volatile compounds during GC. This technique is less easy to handle than conventional SDE. Chaintreau (in press) is a review SDE developments.

Simple steam distillation under vacuum (Forss and Holloway, 1967; Joulain, 1986) may be used with the same goal to avoid thermal artifacts. The distillates (i.e., aromatic water phase) are further extracted by using the appropriate organic solvent. Solvents are then dried, concentrated, and injected onto a GC column.

Volatile traps

The key advantage of this headspace method, compared to static headspace analysis, is the sensitivity obtained. While static headspace shows the status at equilibrium and can measure thermodynamic constants, "purge-and-trap" or dynamic headspace can measure the kinetics of

release by attaching several traps, each corresponding to a different time period (Roberts and Acree, 1995).

In flavor analysis, the most frequent use of volatile traps is in analyzing the flavor compounds in foods using "purge-and-trap" or dynamic headspace, followed by GC-MS or GCO. Additionally, the traps can be used to measure static headspace and air-matrix partition coefficients where air is pushed out of an equilibrated cell containing the sample onto a volatile trap (Chaintreau et al., 1995). Volatile traps have been also used for flavor release measurements during eating (Linforth and Taylor, 1993) or simulated eating (Roberts and Acree, 1995).

While the method described here uses thermal desorption to release the volatiles from the trap directly to the GC, another method less often used is solvent extraction of the trap. Even though this will result in a lower sensitivity as the complete trap contents are diluted in solvent, an advantage is the possibility for multiple injections. Additionally, cold traps rather than adsorbent traps are also used to trap volatiles (Badings et al., 1985) although the water and CO_2 that are also condensed can pose GC difficulties.

One of the key developments in the development of thermal desorption devices was the possibility for cryofocusing systems that have the advantage of "injection-like" samples. A short section of capillary tubing at liquid nitrogen temperatures (i.e., −160°C) traps the volatiles. When capillary columns replaced packed columns as the standard, complete flow from the desorption trap (5 ml/min minimum) to the capillary columns (~1 ml/min) was possible through the use of cryofocusing. The "split injection" interface was another development that splits the flow so that only a part of the desorbed volatiles entered the column. While this allowed the need for cryofocusing to be circumvented, sensitivity was lost due to the split.

Critical Parameters and Troubleshooting

SDE

The temperature of the baths must be precisely adjusted. It is best if these baths are dedicated to these extractions. Likewise, the cold finger condenser must be maintained at ~0°C, usually by the use of a cooling bath. The cooling temperature given by tap water (generally between 10° and 15°C) is not enough to insure a good condensation of volatile aroma, and losses may occur.

The choice of the solvent depends on the polarity of compounds contained in the product. Solvents such as dichloromethane, diethyl ether, and pentane are often chosen because they have low boiling points and are thus rapidly eluted in the GC and are easy to concentrate (Maignial et al., 1992).

In the case of foaming materials, the distillation must be carefully watched. Additional antifoaming agents can be used, but they will be coextracted and may cover some sample volatiles on the GC profile. Their use is not recommended.

Volatile traps

Sampling. The flow rate and time of trapping should be determined experimentally. Typically, flow rates in the range of 10 to 80 ml/min are used along with purge times on the order of 10 to 60 min. The flow rate can affect the type of compounds that are stripped, with highly volatile compounds being stripped with higher flow rates. An optimization study for tomato volatiles found a 20 ml/min optimum when using a 60-min trap time (Sucan and Russell, 1997). Another critical parameter to check is breakthrough. As time or flow rate is increased, compounds with higher affinity for the adsorbent material may displace other compounds. This will result in a distorted volatile profile. When performing the optimization studies, it is wise to begin with a less concentrated sample and evaluate the chromatography before testing the adsorption limits of the trap. This is to avoid system contamination. Even trace contamination of the traps and transfer lines causes large difficulties for GCO. If using pure standards, do so at dilute concentrations (i.e., ppm or lowest level needed for a reliable signal).

For the vessel purging, it is recommended that nitrogen or helium gas be used, rather than air, due to oxidation of the food product. The temperature chosen depends on the purpose of the experiment. Room temperature is often chosen to simulate volatile emanating from the product in its natural state. Slightly elevated temperatures (37°C) may better simulate flavor release from the mouth. Higher temperatures should not be used for samples that change with temperature and are normally consumed at lower temperatures, unless the purpose is to see the volatiles released upon heating. Temperatures over 60°C often result in too much water being trapped, which affects gas chromatographic separation and mass spectrometric

detection when in splitless mode. If sensitivity is not an objective, split mode can be used to reduce the water on the column. Stirring is another parameter that can be included if, for example, the study's purpose is to understand the volatiles released upon chewing. After trapping, thermal desorption normally should take place immediately afterwards, although effective trap caps could add stability over time.

The sample vessel should be tested to ensure that it is leak-proof during purging with the trap attached. For example, this can be done by applying soapy water at joints. Instead of purging gas through the system, using a vacuum pump that is attached to the outlet of the trap, with a fitting allowing filtered air to enter, is another option to solve leaks (Wampler, 1997).

In some applications of purge-and-trap, foaming can be a problem. Possible solutions include using a bubble breaker or foam filtration at the top of the purging vessel (Wampler, 1997), a needle sparger, or using dynamic headspace analysis instead. Use of an antifoam emulsion can also help, although this will add some impurities and may change the flavor release, as these emulsions are oil-based.

Adsorbent choice. The choice of adsorbent material depends on the volatile compounds in the food. Of the synthetic porous polymers, the most widely used and best overall adsorbent is Tenax TA (poly-2,6-diphenyl-p-phenylene oxide) 60 to 80 mesh. While Tenax does not show an adsorption capacity for all volatiles, especially very small polar compounds such as acetaldehyde, it has good thermal stability and desorption capabilities. It also traps little water and generates very few artifacts. Table G1.2.2 shows a few limitations and advantages of various adsorbents, all of which can be purchased from chromatography suppliers. If very small volatiles are the goal, various Carbosieves could be used, or traps containing several adsorbents in series. Traps with mixed adsorbents should be desorbed immediately, before transfer between phases occurs.

Trap desorption. The choice of the thermal desorption apparatus is critical in order to avoid contamination and to be able to work with aroma compounds in a wide range of retention indices. In all systems, problems can be encountered due to reactive compounds or cold spots within the analyzer. It is recommended that all transfer lines, valves, or surfaces in contact with the volatile compounds be made of an inert material such as fused-silica or deactivated glass-lined stainless steel. Even more ideal are systems that do not have long transfer lines. Some thermal desorption systems offer a water removal system, which should not be used for recovery of flavor compounds, some of which are of low volatility and hydrophilic. Additionally, it is not appropriate to analyze thermally-labile compounds by thermal desorption of traps.

Anticipated Results

SDE

Very high sensitivity (to ppt) can be obtained with compounds that are extractable by SDE. However, highly water-soluble compounds are not extracted completely and compounds with very low boiling points, such as methanethiol, can be lost during concentration. In addition, compounds with gas chromatographic elutions simultaneously with the solvent are hidden from view. Quantification can be made by addition of an internal standard to the sample matrix.

Volatile traps

While the exact recovery depends on the volatility of the flavor compounds, most compounds can be detected with this method when present at ppb (mg/liter) concentrations. Reproducibility (CV) is between 5% and 10%. To quantify the amount trapped, an internal standard curve can be made by adding the standards in solvent directly to the trap just before thermal desorption on the side of gas entry during thermal desorption. For liquid homogeneous samples, quantification of the amount in the matrix can be done by a standard addition methodology.

Time Considerations

For SDE, a well-prepared scientist needs between 2 and 3 hr to perform the whole extraction, including the concentration. The extraction must be surveyed for ~20 min until the distillation reaches a constant rate.

For volatile trapping, preparation of the traps and conditioning requires ~1 hr. Trapping of the aroma compounds requires ~15 min. Thermal desorption of the traps followed by GC analysis requires ~1 hr.

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Key References

SDE

Chaintreau, in press. See above.

Review of the evolution of SDE from the original design to the version operating under vacuum. Comparison of SDE with other extraction techniques are presented.

Forss and Holloway, 1967. See above.

Three processes were compared for volatile extraction from fat matrix: high vacuum degassing, cold-finger molecular distillation, and reduced pressure steam distillation.

Godefroot et al., 1981. See above.

The diagram of the micro steam distillation-extraction apparatus is given. The recovery yield is given for test compounds.

Maignial et al., 1992. See above.

Diagrams of SDE under vacuum and solvent consideration are given. The reader can also consult the additional literature giving other details relative to the methodology: i.e., the Chrompack user manual contains a drawing of themicro–steam distillation extraction apparatus.

McGill, A.S. and Hardy, R. 1977. Artefact production in the Likens-Nickerson apparatus when used to extract the volatile flavourous components of cod. *J. Sci. Food Agric.* 28:89-92.

Artifact formation during the extraction is demonstrated. The effect of N_2 flushing and of antioxidant addition is shown.

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The authors show differences in aroma profiles and odor evaluation due to the effect of temperature.

Romer, G. and Renner, E. 1974. Simple methods for isolation and concentration of flavor compounds from foods. *Z. Lebensm. Unters. Forsch.* 156:329-335.

The design of a new apparatus is given. It is made for the extraction of larger quantities of material.

Schultz et al., 1977. See above.

The diagram of the apparatus is given. The effect of pH and the recovery yield in function of time are described.

Volatile traps

Abeel, S.M., Vickers, A.K., and Decker, D. 1994. Trends in purge and trap. *J. Chromatogr. Sci.* 32:328-338.

Extensive review article of purge-and-trap techniques geared towards environmental applications. Good discussion of choice of extraction conditions including purge rate and time.

Sucan and Russell, 1997. See above.

Example of purge-and-trap optimization of conditions.

Wampler, 1997. See above.

In depth discussion of many aspects related to headspace sampling.

Contributed by Deborah Roberts and Hugues Brevard
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Identification and Quantitation of Aroma Compounds

After isolation of the volatiles using different extraction and concentration procedures (*UNIT G1.2*) the samples are injected into a gas chromatograph (GC) for separation of individual compounds. Identification is based on their Kovats retention indices on two columns of different polarity, as well as comparison with mass spectroscopy (MS) spectra of the corresponding reference compounds. Quantification is then performed using one or several compounds as internal standards, which naturally do not occur in the sample, added to the sample before extraction, if possible. Depending on the type and availability of these standards, quantitation is preferably performed by gas chromatography-flame ionization detection (GC-FID; see Basic Protocol) or gas chromatography-mass spectrometry (GC-MS) using isotope dilution assays (IDAs; see Alternate Protocol). Other methods for quantification based on external standards are less accurate, and those based on multiple standard addition at different concentrations are only rarely needed.

IDENTIFICATION (GC-FID; GC-MS) AND QUANTIFICATION OF VOLATILES (GC-FID)

BASIC PROTOCOL

Volatiles are separated by GC and their elution time monitored relative to a series of *n*-alkanes that are injected under identical conditions. The most common GC detector is the flame ionization detector (FID), which is also widely used in flavor research. The detector responds to all organic compounds that burn or ionize in its flame, and is characterized by a large dynamic range (Buffington and Wilson, 1987). The temperature program chosen for the separation of compounds is dominated by mutually exclusive parameters like speed, resolution, and capacity. For separation of highly volatile odorants, sub-ambient GC-start temperatures are recommended. The steps described in this unit for the identification are limited to compounds that are listed in common data banks. Analytical methods to identify a compound that has never been reported before are not discussed, and the reader is referred to experts in natural products chemistry. Unambiguous identification requires that an authentic standard has been shown to have the same chromatographic, spectral, and olfactory properties. Anything less is tentative; however, like other problems in analytical biochemistry the biological activity (i.e., olfaction) is a powerful indication of the identity of a standard and an analyte especially when combined with chromatography.

Materials

Aroma samples
Solution of 10 to 100 µg/ml *n*-alkanes (C5 to C25) in diethylether (freshly distilled)
Reference compound
Internal standard compound

Gas chromatograph with FID and sniffing port (Hewlett Packard)
DB5 and DBWAX capillary columns (e.g., 30-m × 0.25-mm; f = 0.25 µm; J&W Scientific)
Gas chromatograph with MS detector (with EI mode and optional CI mode; Hewlett Packard)

Additional reagents and equipment for extraction methods (see *UNIT G1.2*)

Contributed by Christian Milo
Current Protocols in Food Analytical Chemistry (2001) G1.3.1-G1.3.12

Identify compounds

1. Inject a blank sample into the GC, equipped with an FID and sniffing port, under the analytical conditions chosen for the aroma sample.

   Most odorants will elute between 700 and 1600 n-paraffin retention indices from non-polar columns like the DB5.

   For aroma extracts, the blank sample is a mixture of the solvents used in the extraction, and are concentrated in the same way as the aroma isolate. Some volatiles in aroma extracts may derive from trace impurities of the solvents. For headspace techniques, a blank run is also recommended to check impurities coming from the tubings and/or adsorbents used.

2. Inject the aroma sample (0.5 µl per solvent extract) in the on-column or splitless mode and simultaneously start the GC sniffing.

   For both on-column and splitless injections of solvent extracts the initial temperature of the GC oven is held slightly above the boiling point of the solvent for 1 to 2 min. After very rapid heating to 50°C to 60°C the heating rate usually is 4°C or 6°C per minute up to the final temperature of 220°C to 240°C for DBWAX columns and up to 250° to 280°C for DB5 capillary columns.

3. Inject the mixture of 10 to 100 µg/ml *n*-alkanes.

   The injection mode (e.g., headspace or liquid injection) should be the same as for the aroma sample.

4. Determine the retention time of the alkanes and of the peaks to be identified in the flavor sample and calculate their retention indices according to the following equation (Van den Dool and Kratz, 1963):

$$RI = 100 \times n(\mathrm{C}) + 100 \times [T_{(a)} - Tn_{(CS)}/Tn_{(CL)} - T_{(a)}]$$

   where n(C) is the number of carbons; $T_{(a)}$ is the retention time of the compound of interest; $Tn_{(CS)}$ is the retention time of smaller hydrocarbons, i.e., eluting before the compound of interest; and $Tn_{(CL)}$ is the retention time of larger hydrocarbon, i.e., eluting after the compound of interest.

   This is shown in Figure G1.3.1.

5. Compare RI indices and aroma quality as assessed during GC sniffing (Acree et al., 1984; Ullrich and Grosch, 1987) with those in the literature (Kondjoyan and Berdagué, 1996), and the Flavornet (*http://www.nysaes.cornell.edu/flavornet*).

   This will give a list of possible candidates matching the RI and sniffing criteria.

6. Repeat steps 1 to 5 on a column of different polarity.

   A DBWAX and DB5 column are recommended because most literature data is available on these phases and they represent a polar column (DBWAX) and an apolar column (DB5), which separate compounds differently.

7. Inject the aroma sample on the same types of columns (i.e., DBWAX and DB5) using an MS detector.

   Focus identification on the odor-active regions as determined by GC-sniffing and RI values as published in the Flavornet (http://www.nysaes.cornell.edu/flavornet). The fragmentation pattern obtained for the compounds can be compared with those in data banks like the Wiley/NBS Registry of Mass Spectral data (McLafferty and Stauffer, 2000) or the NIST 98 library (National Institute of Science and Technology).

8. Inject reference compound.

   An aroma compound is unambiguously identified only when aroma quality, the GC retention indices, and the MS spectra are the same as for the reference compound.

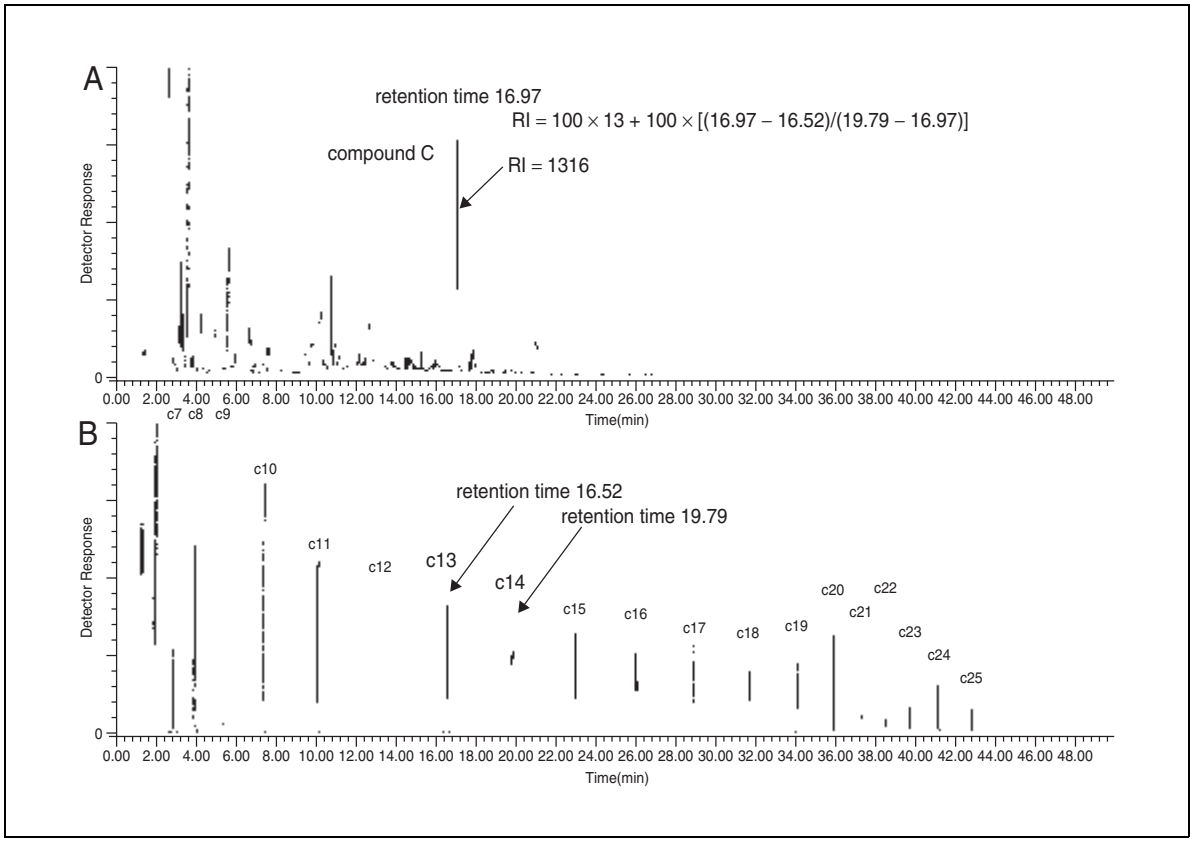

Figure G1.3.1 Determination of the retention index (RI) of an unknown compound C in an aroma extract (**A**) by comparing with a series on *n*-alkanes (**B**) analyzed under the same GC-conditions.

Quantify using GC-FID

9. Choose a compound as an internal standard.

   The standard should have similar chemical and physical properties as the compound that is to be determined (e.g., benzylthiol for quantification of furfurylthiol). It needs to be naturally absent in the sample and must not coelute with other sample components.

10. Add an appropriate amount of this standard preferably a liquid or a suspension of finely ground solid material to the sample.

    The concentration of the standard should be in the same order of magnitude as the analyte.

11. Work up sample by extraction methods (see *UNIT G1.2*) or headspace sampling (*UNIT G1.2*; Milo and Grosch, 1995; Roberts et al., 2000).

12. Inject sample into GC under the same analytical conditions as for the previously described identification steps.

13. Calculate concentration of analyte (absolute amount of A) according to the following equation:

$$C_{(A)} = F_{(A)}/F_{(ST)} \times C_{(ST)} \times f_{resp} \times f_{rec}$$

where $F_{(A)}$ is the peak area of analyte; $F_{(ST)}$ is the peak area of standard; $C_{(ST)}$ is the amount of standard added; f_{resp} is the FID response factor; and f_{rec} is the recovery factor.

Sometimes the determination of FID response factors may not be necessary, especially if the standard has a similar molecular formula as the analyte of interest and therefore is close to 1.

14. Repeat steps 10 to 13 in a model system containing known amounts of analyte and standard to determine the relative recovery factors of analyte versus standard throughout the isolation procedure.

The ratio of the analyte to the standard added, divided by the ratio of the analyte to the standard measured, is the recovery factor. Multiplying the resulting measurements by the appropriate recovery factor will improve accuracy by reducing some of the matrix effects.

ALTERNATE PROTOCOL

QUANTIFICATION OF AROMA COMPOUNDS BY ISOTOPE DILUTION ASSAY (IDA)

Aroma compounds can be quantified with high accuracy by using their corresponding isotope labeled analogs as internal standards, e.g., the concentration of dimethylsulfide in a product will be determined with d6-dimethylsulfide. Due to the almost identical chemical as well as physical properties all possible losses of the analyte during the isolation, extraction, and concentration steps are fully compensated for. Since many standards are not available solvent-free in a high enough purity, the procedure involves (1) the determination of the concentration of labeled standards, (2) homogenization of isotopically labeled standard throughout the sample, and (3) GC-MS analysis in different MS modes depending on the nature of the compound (fragmentation pattern) and sensitivity needed. Although chemical ionization in positive and negative mode may have certain advantages in terms of selectivity and sensitivity, electron impact ionization for MS is sufficient for many cases.

Materials

Isotope labeled standards: a few aroma volatiles are commercially available from CDN Isotopes and Cambridge Isotope Laboratories (CIL); custom synthesis is available from Aspen Research Laboratories and Aldrich; the standards may also be synthesized in-house
Purified aroma compounds to be quantified
Ester of high GC purity (e.g., methyloctanoate)
Organic solvents (highest purity)
Food sample to be analyzed

Extraction apparatus or headspace adsorption device
Gas chromatograph with MS detector (EI mode and optional CI mode; Hewlett-Packard)
Gas chromatograph with FID (Hewlett-Packard)

NOTE: Store standards highly diluted (ppm) in organic solvents, preferably CH_2Cl_2 or methanol (for thiols) and in a freezer. Compounds like vinylguaiacol or furfurylthiol are stable under these conditions.

NOTE: Solution of labeled standards stored in the freezer should be brought up to room temperature before opening the vials and using them. If standard solutions are in a very low-boiling solvent, e.g., pentane, use them refrigerated to facilitate pipetting.

Determine purity and concentration of labeled standard for non-commercially available standards by GC-FID

1. Inject 0.5 µl of a solution containing the labeled standard (A*) at a concentration of ~0.005% and determine the purity of the solution.

Possible impurities in the standard solutions should be identified by GC-MS. Impurities can be tolerated if they do not interfere with the quantitation.

2. Determine the GC-FID response of a known amount of the unlabeled aroma compound ($C_{(A)}$) and an ester ($C_{(E)}$) of high GC purity (e.g., methyloctanoate) that does not coelute with any impurities coming from the labeled standard solution. Calculate the response factor as follows:

$$f_{resp} = C_{(A)}/C_{(E)} \times F_{(E)}/F_{(A)}$$

where C indicates concentration and F is the peak area.

3. Take a 200-µl aliquot of labeled standard solution (A*) and 200 µl of ester (E) in approximately the same concentration and determine the exact concentration of the labeled standard by peak area comparison correcting for the previously determined response factor according to the equation:

$$C_{(A^*)} = F_{(A^*)}/F_{(E)} \times C_{(E)} \times f_{resp}$$

where $C_{(A^*)}$ is the concentration of labeled standard; $F_{(A^*)}$ is the peak area of labeled standard; $F_{(E)}$ is the peak area of the ester; $C_{(E)}$ is the concentration of the ester; and f_{resp} is the response factor [$F_{resp} = C_{(A)}/C_{(E)} \times F_{(E)}/F_{(A)}$].

The concentrations of these standard solutions should be checked regularly depending on the expected chemical stability.

Add standards

The sample should be liquid or a slurry of a finely ground solid sample.

4. Add an aliquot (10 to 500 µl) of the organic solution containing the labeled standard, keeping the ratio of analyte (A) in the food sample and its labeled analog (A*) in the range of 0.2- to 5-fold.

The volume of standard added should be kept to a minimum if headspace sampling or SPME sampling is performed. For extraction work-up procedures, the amount of solvent added via the standard may not be critical at all.

5. Stir the solution vigorously for 30 min after adding the standard and before proceeding with the work-up.
6. Transfer the sample to an extraction apparatus or headspace adsorption device for the isolation of volatiles.

Quantify by GC-MS

7. Inject 0.5 µl of a solution containing the labeled standard (A*) at a concentration of ~0.005% by GC-MS (EI mode).
8. Study the fragmentation pattern of the labeled compound and compare it with the compound to be quantified.
9. Choose selective mass traces of labeled and unlabeled compound for subsequent quantification using single ion monitoring (SIM mode).

The mass traces of the unlabeled and labeled counterparts should be specific and preferably of a high intensity. For compounds showing a strong molecular ion in MS-EI the respective molecular mass ions will be used.

In the absence of a strong molecular ion, and if the EI-spectra of labeled standard and analyte are too, other similar ionization modes need to be considered (CI positive/negative; different reactant gases).

10. Establish a calibration curve, based on selected mass traces, by injecting a series of solutions containing the same amount of labeled standard and varying amounts of the analyte over a concentration range of 0.2- to 5-fold.

To obtain the calibration curve, the amount ratio of unlabeled compound/labeled compound (x-axis) is plotted against the ratio of peak area of the mass trace of the unlabeled

compound/peak area of the mass trace of the labeled compound (y-axis). The slope of the curve represents the response factor. A linear calibration curve is usually obtained within a small range of concentration (0.2- to 5-fold) and if there is no overlapping of the mass traces chosen for the unlabeled and labeled compounds. In cases of nonlinear calibration curves, linearizations can be performed and the reader is referred to the literature (Fay et al., 2000).

11. Inject the aroma extract/sample spiked with the standard, prepared in steps 4 to 6.

12. Determine the amount of analyte in the aroma sample by GC-MS via the peak areas of the selected mass traces according to the equation:

$$C_{(AS)} = C_{(A^*)} \times F_{(AS)}/F_{(A^*)} \times R_{(MS)}$$

where $C_{(AS)}$ is the amount of analyte in the sample; $F_{(AS)}$ is the peak area of analyte mass trace; $C_{(A^*)}$ is the concentration of labeled standard; $F_{(A^*)}$ is the peak area of labeled standard mass trace; and $R_{(MS)}$ is the MS response factor as determined in step 10.

COMMENTARY

Background Information

Identification (GC; GC-MS) and quantification of volatiles (GC-FID)

As perception of most aroma depends on a subtle balance of different odorants, understanding complex aroma at the molecular level means focusing on sensorially relevant odorants.

The identification of aroma-active volatiles targets the odor-active regions in the GC chromatograms. Determined by GC-sniffing and further characterized by the retention indices on columns of different polarity, not all potent odorants will give an FID signal. Their concentration in the extract may be too low to produce an FID signal but may still be sniffed due to the high sensitivity of human olfaction to certain odorants. This interferes with identification, requiring work up of higher sample quantities. Furthermore it means that the match of the retention index of an odor perception during GC-sniffing to an MS signal may be due to coelution with a seemingly pure but odorless component. Identification, as always, requires comparison with reference compounds that may need to be synthesized because they are not commercially available. Information about the occurrence of heteroatoms in a complex aroma extract can be obtained using element-specific detectors, like the flame photometric detector (FPD) for sulfur compounds and the nitrogen phosphorus detector (NPD) for nitrogen and phosphorus compounds. Besides their high selectivity for certain heteroatoms, these detectors are also more sensitive than the FID (Buffington and Wilson, 1987). A rather new detector, the atomic emission detector (AED), allows the simultaneous recording of several elements like C, N, and S in one run, is highly sensitive and has a high linear range for S-compounds. A comparison of different detectors for the analysis of S-containing volatiles showed the strength of the AED compared to the other detectors (Mistry et al., 1994).

Reliable quantitative data are a prerequisite for evaluating the contribution of a single odorant to a positive aroma or off-flavor. They are needed to calculate odor activity values (OAV) that are defined as the ratio of the concentration to the sensory threshold of a given compound in a matrix (Rothe and Thomas, 1963; Acree et al., 1984). These values give guidance in the evaluation of the impact of an odorant to the overall aroma profile.

The main advantage of quantification using internal standards and GC-FID is the simple, inexpensive instrumental set-up as well as the availability of standards compared to IDA.

Quantitation using unlabeled compounds as internal standards and GC-FID detection lacks the high sensitivity and the high selectivity required for aroma compounds present in the ppb level. For chemically stable compounds and those in higher concentration (i.e., >1000 ppb), however, this method gives reliable data. In all other cases, using isotope labeled compounds as internal standards is the method of choice if the they are available.

Quantification of aroma compounds by isotope dilution assays

The use of stable isotope labeled compounds as internal standards requires MS detection

Table G1.3.1 Important Aroma Compounds, Their Labeled Analogs, and Literature References That Use The Technique in Flavor Research

Aroma compound	Isotope label for standard	Literature references
Acetylpyrazine	$[^2H_3]$acetyl	Schieberle and Grosch, 1987
2-Acetyl-1-pyrroline	$[^2H_{2\text{-}7}]$	Schieberle and Grosch, 1987
2-Acetyl-2-thiazoline	$[^2H_4]$	Cerny and Grosch, 1993
2-Acetyltetrahydropyridine	$[^2H_{2\text{-}5}]$	Schieberle, 1995b
2-Aminoacetophenone	$[^2H_3]$aceto	Dollmann et al., 1996
Bis(2-methyl-3-furyl)disulfide	Bis(2-$[^2H_2]$methyl)-	Sen and Grosch, 1991
2,3-Butanedione	$[^{13}C_4]$	Schieberle and Hofmann, 1997
Butanoic acid	3,4-$[^2H_{3\text{-}4}]$	Schieberle et al., 1993
Coumarin	$[^{13}C_2]$	Masanetz and Grosch, 1998a
(E)-β-damascenone	$[^2H_6]$	Sen et al., 1991
(E,E)-2,4-decadienal	3,4-$[^2H_2]$	Lin et al., 1999a
δ-Decalactone	$[^2H_5]$	Milo and Blank, 1998
Decanoic acid	4,5-$[^2H_{2\text{-}4}]$	Guth, 1997
(Z)-6-Decenal	$[^2H_{6\text{-}8}]$	Masanetz and Grosch, 1998b
2,3-Diethyl-5-methylpyrazine	$[^2H_3]$5-methyl	Cerny and Grosch, 1993
Dimethyltrisulfide	$[^2H_6]$	Milo and Grosch, 1996
(Z)-6-dodeceno-y-lactone	6,7-$[^2H_2]$	Schieberle et al., 1993
trans-4,5-Epoxy-(E)-2-decenal	4,5-$[^2H_2]$	Lin et al., 1999
trans-2,3-Epoxyoctanal	$[^2H_?]$	Guth and Grosch, 1993b
2-Ethenyl-3,5-dimethylpyrazine	$[^2H_3]$	Mayer et al., 1999
Ethyl anthranilate	$[^2H_3]$ethyl	Aubry et al., 1997
Ethyl cinnamate	$[^2H_3]$ethyl	Aubry et al., 1997
Ethyl cyclohexanoate	$[^2H_3]$ethyl	Guth and Grosch, 1993c
Ethyl dihydro cinnamate	$[^2H_3]$	Aubry et al., 1997
Ethylguaiacol	$[^2H_3]$methoxy	Semmelroch et al., 1995
5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	$[^2H_3]$ethyl	Blank et al., 1993
5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone (homofuraneol)	$[^2H_3]$ethyl	Blank et al., 1997
Ethyl 2-methylbutanoate	2,2,2-$[^2H_3]$	Guth and Grosch, 1993c
Ethyl 3-methylbutanoate	2,2,2-$[^2H_3]$	Guth, 1997
2-Ethyl-3,5-dimethylpyrazine	$[^2H_3]$	Cerny and Grosch, 1993
3-Ethylphenol	$[1,1\text{-}^2H_2]$	Guth, 1997
2-Furfurylthiol	$[\alpha\text{-}^2H_2]$	Sen and Grosch, 1991
Geosmin	$[^2H_3]$	Palmantier et al., 1998
2-Heptanone	6,7-$[^2H_2]$	Preininger and Grosch, 1994
(Z)-4-Heptenal	$[^2H_?]$	Widder and Grosch, 1994
Hexanoic acid	$[^2H_2]$	Jagella and Grosch, 1999
Hexanal	5,6-$[^2H_2]$	Lin et al., 1999a
(E)-2-Hexenal	$[^2H_?]$	Guth and Grosch, 1993c
(Z)-3-Hexenal	$[^2H_?]$	Guth and Grosch, 1990
(Z)-3-Hexenol	3,4-$[^2H_2]$	Guth and Grosch, 1990
(Z)-3-Hexenyl acetate	$[^2H_3]$acetate	Guth and Grosch, 1993c
4-Hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol)	5,6-$[^{13}C_2]$	Blank et al., 1997
3-Hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone)	5,6-$[^{13}C_2]$	Blank et al., 1996
4-Hydroxy-2-nonenoic acid lactone	$[^2H_?]$	Guth and Grosch, 1993b
2-Isobutyl-3-methoxypyrazine	$[^2H_3]$methoxy	Semmelroch and Grosch, 1996

continued

Table G1.3.1 Important Aroma Compounds, Their Labeled Analogs, and Literature References That Use The Technique in Flavor Research, continued

Aroma compound	Isotope label for standard	Literature references
2-sec-Butyl-3-methoxypyrazine	[$^2H_3$]methoxy	Rychlik et al., 1997
2-Isopropyl-3-methoxypyrazine	[$^2H_3$]methoxy	Masanetz and Grosch, 1998b
p-Mentha-1,3,8-triene	[$^2H_3$]	Masanetz and Grosch, 1998b
3-Mercapto-2-butanone	[$^{13}C_4$]	Schieberle and Hofmann, 1996
3-Mercapto-3-methylbutyl formate	[$^2H_6$]	Masanetz et al., 1995
4-Mercapto-4-methyl-pentan-2-one	[$^{13}C_4$]	Guth, 1997
3-Mercapto-2-pentanone	[4,5-$^2H_2$]	Sen and Grosch, 1991
Methanthiol	[$^2H_3$]	Guth and Grosch, 1994
Methional	[$^2H_3$]methyl	Sen and Grosch, 1991
4-Methoxy-2,5-dimethyl-3(2H)-furanone	[$^2H_3$]methoxy	Schieberle and Hofmann, 1997
4-Methoxy-2-methyl-2-butanethiol	[$^2H_3$]methoxy	Guth and Grosch, 1993c
2-Methoxyphenol (guaiacol)	[$^2H_3$]methoxy	Cerny and Grosch, 1993
Methylanthranilate	[$^2H_3$]	Aubry et al., 1997
3-Methylbutanal	[$^2H_2$]	Schieberle and Grosch, 1992
3-Methylbutanol	[$^2H_{2-5}$]	Schieberle, 1991
3-Methyl-2-butene-1-thiol	[$^2H_8$]	Semmelroch and Grosch, 1996
3-Methylbutyric acid	3,4-[$^2H_2$]	Guth and Grosch, 1994
5-Methyl-5H-cyclopenta(b)pyrazine	[$^2H_{1-2}$]	Schieberle and Grosch, 1987
2-Methyl-3-furanthiol	[$^2H_3$]methyl	Sen and Grosch, 1991
5-Methyl-(E)-2-hepten-4-one	6,7-[$^2H_2$]	Pfnuer et al., 1999
2-Methylisoborneol	2-[$^2H_3$]	Palmantier et al., 1998
3-Methyl-2,4-nonandione	3-[$^2H_3$]methyl	Guth and Grosch, 1990
Methylpropanal	[$^2H_7$]	Milo and Grosch, 1996
12-Methyltridecanal	12-[$^2H_{3-8}$]	Guth and Grosch, 1993a
Myristicin	[$^2H_2$]	Masanetz and Grosch, 1998b
(E,E)-2,4-Nonadienal	3,4-[$^2H_2$]	Lin et al., 1999a
(E,Z)-2,6-Nonadienal	6,7-[$^2H_2$]	Fielder and Rowan, 1999
Nonanal	3,3,4,4-[$^2H_4$]	Fielder and Rowan, 1999
(Z,Z)-3,6-Nonadienal	[$^2H_4$]	Milo and Grosch, 1993
(E)-2-Nonenal	2,3-[$^2H_2$]	Lin et al., 1999a
(Z)-2-Nonenal	[$^2H_2$]	Guth and Grosch, 1990
(Z)-1,5-Octadiene-3-one	5,6-[$^2H_2$]	Lin et al., 1999b
1-Octen-3-hydroperoxide	[$^2H_2$]	Guth and Grosch, 1990
1-Octen-3-one	1,1,2-[$^2H_{2-3}$]	Lin et al., 1999b
2,3-Pentanedione	5,5,5-[$^2H_3$]	Milo and Grosch, 1993
Pentanoic acid	[$^2H_3$]	Jagella and Grosch, 1999
2-Pentylpyridine	[$^2H_4$]	Schieberle, 1996
2-Phenylethanol	1,1-[$^2H_2$]	Schieberle, 1991
2-Phenylethyl acetate	1,2-[$^{13}C_2$] acetate	Guth, 1997
2-Phenylethylthiol	[α-$^2H_2$]	Schieberle, 1996
cis-Rose oxide	[$^2H_2$]	Guth, 1997
Methylindol (skatol)	[$^2H_3$]-methyl	Preininger and Grosch, 1994
2,4,5-Trimethylthiazol	[5-methyl-$^2H_3$]-	Sen and Grosch, 1991
Vanillin	[$^2H_3$]-methoxy	Semmelroch et al., 1995
4-Vinylguaiacol	[$^2H_3$]-methoxy	Semmelroch et al., 1995
Wine lactone	[$^2H_3$]	Guth, 1997

since their very similar chemical and physical properties cause them to coelute with the compound to be quantified. The isotope dilution assay is the most accurate method currently available for the quantification of labile odorants and those in low concentrations. After the first application of IDA for flavor research (Schieberle and Grosch, 1987) the method was systematically developed for >60 potent odorants (Schieberle, 1995a). The accuracy of IDAs and quantification with unlabeled internal standards was recently discussed (Preininger, 1998). Table G1.3.1 lists important aroma compounds as well as their labeled analogs and gives an extensive overview of the literature using this technique in flavor research.

One of the main advantages of IDA is that quantitative isolation of odorants from the sample is not required, provided that the internal standard is homogeneously distributed throughout the sample. Solid samples should be finely ground under liquid nitrogen in order to facilitate even penetration of the labeled standard throughout the sample. The time necessary for the standard to be evenly mixed with the analyte can be checked (Milo and Blank, 1998). Due to the high chemical and physical similarities of isotopomers, losses during the work up procedure are ideally compensated. Another strength of the method is the high selectivity and sensitivity of GC-MS, particularly in selected ion monitoring mode. Compounds that are poorly resolved by GC may not interfere in GC-MS due to different fragmentation patterns and, therefore, less sample clean-up is needed (and under CI less separation is needed).

The main hurdle to the application of IDA for quantitative measurements of aroma compounds continues to be the limited commercial availability of the labeled internal standards.

Critical Parameters and Troubleshooting

Identification and quantification of volatiles (GC-FID)

The most critical parameter for the Basic Protocol is the yield of analyte at the detector. Simulations and models are the best methods to identify the extent of the problem and to determine correction factors.

Quantification of aroma compounds by isotope dilution assays

The labeled standards used need to be isotopically stable. Isotopic stability may become an issue if the compound is labeled with deuterium in an enolizable position as is the case for the α-position of carbonyl functions. The deuterium may then be exchanged with protons from the sample during the workup and therefore falsify the result. In order to rule out such D/H exchanges, the standard may be tested under the conditions of the isolation of volatiles from the sample or, better, be replaced by a standard labeled with ^{13}C.

Furthermore the labeling of the compound should increase its molecular weight by at least 2 units, preferably 3 units, in order to minimize interferences with the natural isotope distribution of the analyte.

For non–commercially available standards, the chemical purity plays an important role. The price for a custom synthesis may be lower if only 70% chemical purity is negotiated. As long as the contaminants do not interfere with other compounds to be quantified in the sample and will not convert into the final labeled product during the workup procedure, this can be tolerated.

Although IDA is particularly useful for the quantitation of labile compounds, one needs to pay attention to possible degradation products. The possible conversion of (Z)-3-alkenals to the (E)-2 alkenals will bias results if these compounds are to be quantified simultaneously. Another example are thiols and their dimers that should be quantified in separate samples using the respective labeled standards.

The EI mode can be used in IDA, provided that characteristic ions of high intensity are available for quantification. In some cases, however, it is necessary to improve the efficiency of IDA by changing and optimizing the ionization technique as recently demonstrated for epoxy-alkenals (Blank et al., 1999).

Anticipated Results

Due to the bias that can occur in the Basic Protocol, results can be as low as 1% of the correct value for the concentration of a volatile in a sample. Most results are <80% of the correct value. This bias is usually caused by differences in the recovery between the internal standard and the analytes. Choosing an internal standard that is similar to the analyte can reduce this bias but it cannot be completely eliminated unless a separate standard is used for each analyte (see Alternate Protocol). Nevertheless, the Basic Protocol is often sufficiently accurate because the olfactory system, unlike the taste system, is compressive and insensitive to small

changes in concentration (Lawless and Heymann, 1998).

Due to the high sensitivity of the IDA method quantification in the sub-ppb range, the Alternate Protocol can be applied without extensive sample cleanups. Due to the high similarity of labeled internal standard and analyte, the relative recovery of analytes from the matrix, whether oily or aqueous, approaches 100% (Guth and Grosch, 1993c; Schieberle and Hofmann, 1998); most errors come from pipetting. The coefficient of variation for compounds present in ppb quantities is between 5% and 10%.

Time Considerations

The time required for quantification of volatiles by both the Basic Protocol and the Alternate Protocol depends on the isolation/extraction procedure chosen. A complete homogenization of the labeled standards with the sample usually requires not more than 30 min and GC-MS analysis is accomplished within 1 hr. In combination with a high-throughput method like solid-phase microextraction, the GC cycle times (~1 hr) become the limiting factor in the quantification of multiple samples by IDAs.

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Stereodifferentiation of Chiral Odorants Using High-Resolution Gas Chromatography

This unit describes those methods that can differentiate between enantiomers found in foods that contribute to their taste and aroma. These compounds are volatile odorants that are most easily analyzed using enantioselective high resolution–gas chromatography (HRGC). Other methods exist for the separation and analysis of chiral compounds, which include optical methods, liquid and planar chromatography, and electrophoresis, but for food volatiles, gas chromatography has evolved to the point where it is now the cornerstone for the most comprehensive analysis of volatile compounds.

Enantioselective gas chromatography can provide three quite different kinds of information: (1) the amount of each enantiomer present in a food, determined as the enantiomeric purity or the enantiomer excess, and the separation factor α for each pair of enantiomers; (2) enantiospecific sensory evaluation using gas chromatography-olfactometry (GC-O); and (3) data used as part of an authenticity determination.

The most useful results will depend upon α being >1.01 which, in turn, depends upon the selection of the correct chiral stationary phase that will provide optimum resolution of enantiomers.

The protocol selected will be determined by the application under investigation and by the instrumentation available. The following instrument configurations can be used:

1. a single oven containing a chiral column with a flame ionization detector (FID);
2. a single oven containing a chiral column with an olfactometer as the detector (GC-O);
3. a multidimensional system consisting of two columns of different selectivity. The first of these two columns, known as the precolumn, is achiral and is fitted with an FID. The second, known as the analytical column, is a chiral column, configured to receive heart-cut sections from the first column (see Alternate Protocol 1; Gordon et al., 1985; Mosandl et al., 1989; Bernreuther and Schreier, 1991; Wright, 1997) and is fitted with either an FID or an olfactometer.
    a. Two columns are enclosed in a single oven, requiring the temperature program to be the same for both columns, or for the heart-cut fraction to be trapped cryoscopically outside the oven while the temperature program is changed.
    b. Two columns are contained in separate ovens, allowing them to be operated with independent gas flows and temperature programs.
    c. Two columns are contained in separate unconnected instruments. The heart-cut fraction is collected in a cryoscopic trap and reinjected into the second column.

Basic Protocol 1 describes how to measure enantiomer ratios using single-dimensional high-resolution gas chromatography, while Alternate Protocol 1 obtains enantiomer ratios using multidimensional gas chromatography. Basic Protocol 2 gives procedures for obtaining enantiospecific sensory data using gas chromatography olfactometry, whereas Alternate Protocol 2 obtains sensory data by multidimensional gas chromatography. A specific application is chosen in each case, so that the choice of chiral column is appropriate. Once the correct operating parameters have been established, a run will take 15 to 100 min, depending upon the application. Some guidelines for the selection of operating conditions and the selection and care of chiral columns are discussed, followed by a troubleshooting section. No protocol is given for an authenticity determination,

Contributed by Mary G. Chisholm

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because Basic Protocol 1 will usually suffice, and for this application, the determination of an enantiomer composition may be part of a more comprehensive analysis involving several instrumental methods.

BASIC PROTOCOL 1

DETERMINATION OF ENANTIOMER COMPOSITION USING SINGLE-DIMENSIONAL HIGH-RESOLUTION GAS CHROMATOGRAPHY

This method can be used when the enantiomers of interest are not coeluting with other compounds in the sample and when accurate quantitative information is not the highest priority of the analysis. The sample will have been prepared by an extraction method selected from those in *UNIT G1.1* and should have a concentration of 50 to 100 ppm. The identity of the components of the sample should be known from gas chromatography-mass spectrometry (GC-MS) together with their retention indices on the achiral stationary phase. Additional sample cleanup procedures may be needed to ensure the optimum results that are evaluated below:

1. online coupling with an achiral precolumn used in multidimensional gas chromatography (MDGC; see Alternate Protocol 1), part of the instrument configuration;
2. offline high performance liquid chromatography (HPLC) removes nonvolatile materials;
3. preparative gas chromatography, a concentration method, used for the examination of specific low concentration compounds of interest;
4. sampling using solid phase micro-extraction (SPME) for enantiomers with retention indices of 800 to 1400 (nonpolar column) removes all nonvolatile and semivolatile compounds, and most compounds eluting at a retention index (RI) >1400. A fast method of examining the most volatile compounds that can be used to evaluate the need for a more accurate analysis. It is very fast, but reproducibility can be poor;
5. identification of coelution by collecting a chromatogram of the sample on a column with a polar stationary phase. Examine peak areas for consistency on the two columns. Time-consuming, but the best method to check for coelution, which leads to the selection of the best column.

If the order of elution of *R* and *S* isomers is required, this must be determined by comparison of the retention times of an authentic sample with those of the analyte.

Materials

C_7-C_{20} *n*-alkane hydrocarbon standard for a nonpolar column, or C_9-C_{30} for a polar column (hydrocarbon standard; see recipe)

Sample containing an on-column concentration of 20 to 80 ng of the enantiomers of interest

High-resolution gas chromatograph fitted with the appropriate chiral column (e.g., heptakis(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin in poly(14% cyanopropylphenyl/86% dimethylsiloxane)), a stationary phase widely used for the chiral analysis of many food extracts

Hydrogen and air lines

FID detector; on-column injector is preferred

Integrator or access to data collection software to record chromatograms

10-µl syringes

Set operating conditions for an essential oil analysis or food extract

1. Use a 30-m × 0.32-mm i.d. column with 0.25-µm film thickness. Set the following operating parameters and allow to stabilize for 30 min.

   Oven temperature: 40°C for 10 min, 2°C/min to 230°C, hold for 10 min
   Carrier gas: H_2 at a linear velocity of 80 cm/sec
   Injector temperature: 150°C
   Split ratio: 100:1
   FID detector temperature: 225°C
   Sample size: 1 µl.

2. Enable the integrator or software for data collection.
3. Inject 1 µl of the hydrocarbon standard onto the column with a 10-µl syringe.
4. Record the chromatogram as a retention index standard for all runs performed under the same operating conditions (*UNIT G1.1*).
5. Allow the GC to reach equilibrium for the next run.
6. Inject a 1-µl sample of the analyte onto the column with a 10-µl syringe and record its chromatogram.
7. Identify the enantiomers of interest using the chromatogram from the hydrocarbon standard and record their retention times and peak areas from the integration data.

8a. Calculate the percentage of each enantiomer present from Equation G1.4.1.

$$\text{enantiomeric purity } \% = \left[\frac{R}{R+S}\right]\times 100 \text{ and } \left[\frac{S}{R+S}\right]\times 100$$

where R and S represent the areas of the R and S peaks of a pair of enantiomers in the chromatogram.

8b. Calculate the separation factor, α from Equation G1.4.2.

$$\alpha = \frac{t_{R2}}{t_{R1}}$$

where t_{R1} and t_{R2} are the corrected retention times for peak 1 and peak 2, respectively.

If it suspected that the enantiomers of interest are coeluting, a correction to the areas of the affected peaks may be applied. The correction can be determined from a knowledge of the peak areas on a nonpolar and polar achiral column. If the shape of the peak and the areas are the same on both columns, then no coelution is occurring. If the areas are different, then a correction factor can be applied by comparing retention times of the separated enantiomers, and subtracting the areas of those peaks that are coeluting, which were determined from the chromatograms obtained from the achiral columns. It should be emphasized that this is not an appropriate correction to make if accurate quantitative information is required.

ALTERNATE PROTOCOL 1

DETERMINATION OF ENANTIOMER COMPOSITION USING MULTIDIMENSIONAL GAS CHROMATOGRAPHY (MDGC)

This method is used when accurate quantitative information is required and the separation produced by a single column is inadequate and cannot be rectified by reconfiguring the instrument. To justify using the more complex system, there should be a guarantee that it can deliver (1) superior resolution compared to that of a single column and (2) increased quantitative information resulting from the elimination of coelution on the chiral column.

This method requires two separate ovens that are operated independently and contain columns of different selectivities. They are connected by a switching device that can selectively transfer small portions of a chromatogram from the first column to the second. These sections of the chromatogram are known as "heart-cut" samples. The heart-cut sample is trapped and cooled with liquid nitrogen. The first column, the precolumn, has an achiral stationary phase that should give the best possible resolution in areas of the chromatogram where enantiomers elute. The second column, the analytical column, should have a chiral stationary phase that gives the largest possible separation factor for

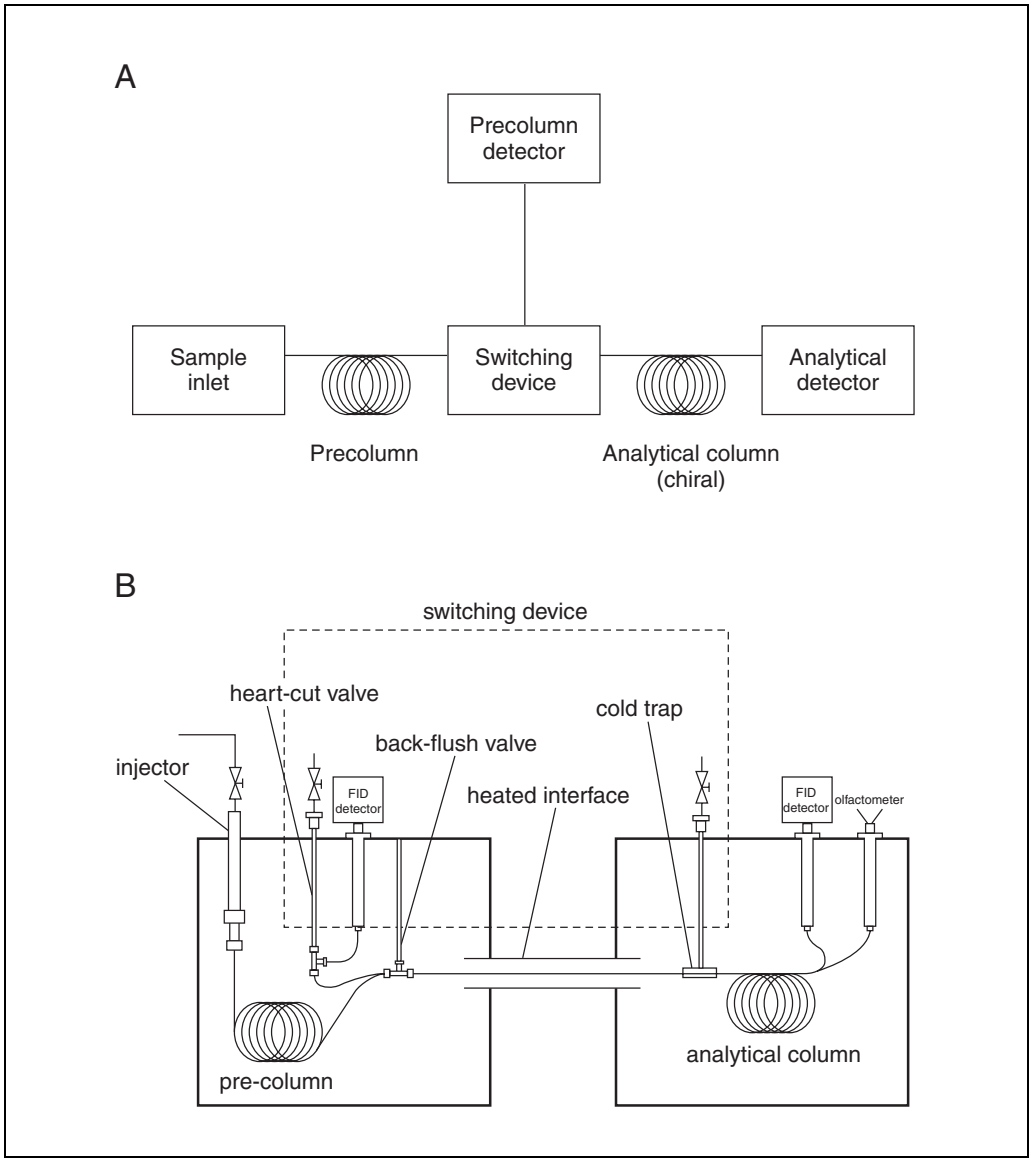

Figure G1.4.1 A two-oven configuration for MDGC. (**A**) The configuration for a basic system. (**B**) A simple system for GC-FID and GC-O showing the essential components (modified from the MDS2000 multidimensional gas chromatography system from SGE; see Table G1.4.3).

the chiral volatiles of interest. Details about specific instrument configurations are in the Commentary section and are shown in Figure G1.4.1. Figures G1.4.2 and G1.4.3 show chromatograms obtained from two different configurations for an MDGC system using the heart-cut technique.

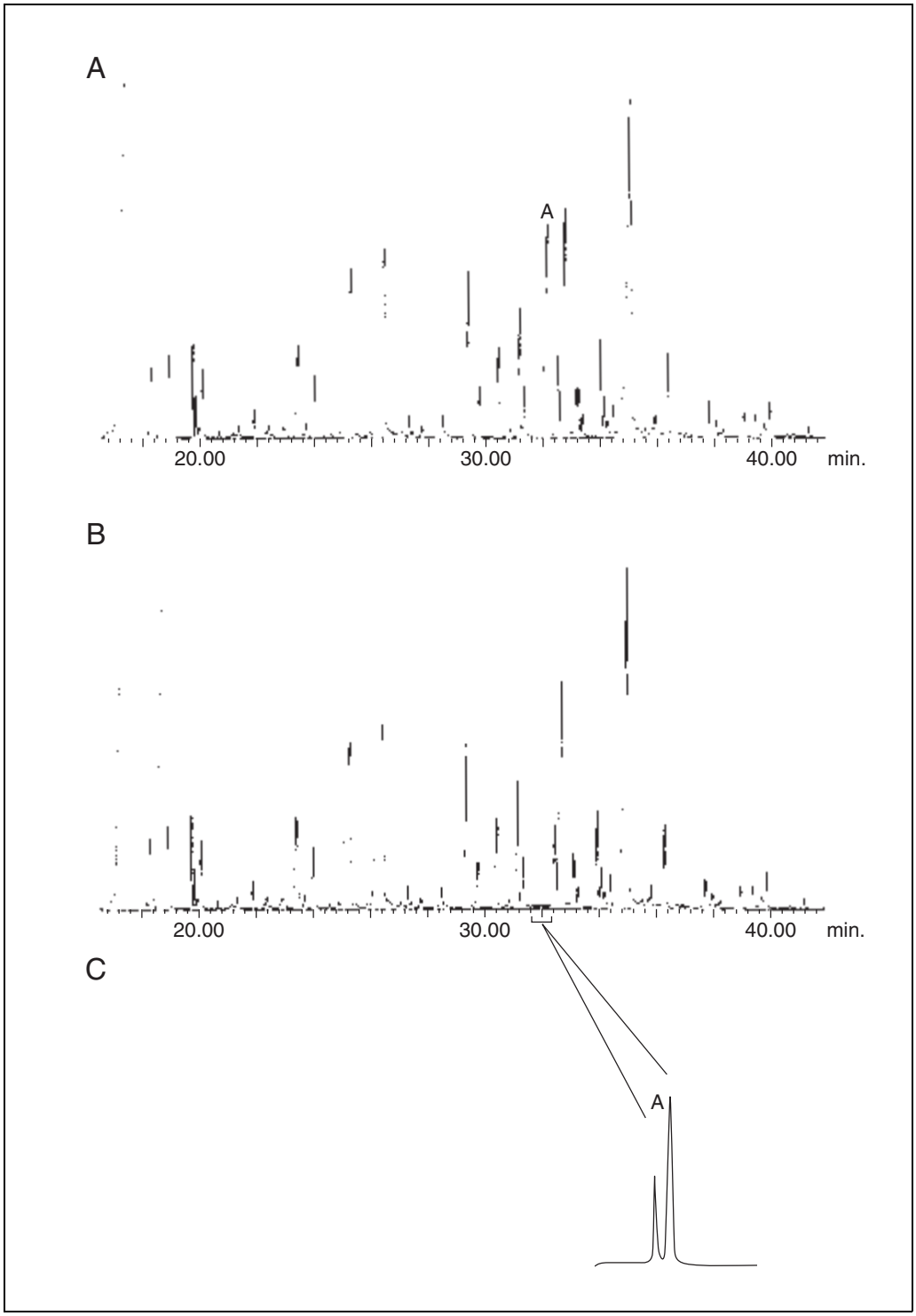

Figure G1.4.2 Chromatograms obtained using a MDGC system. (**A**) Part of a chromatogram obtained from the precolumn (nonpolar) showing the peak of interest A. (**B**) The same sample showing the result of the heart-cut on the precolumn. Peak A is missing. (**C**) The chromatogram obtained from the chiral analytical column, showing the separation of the *R* and *S* isomers.

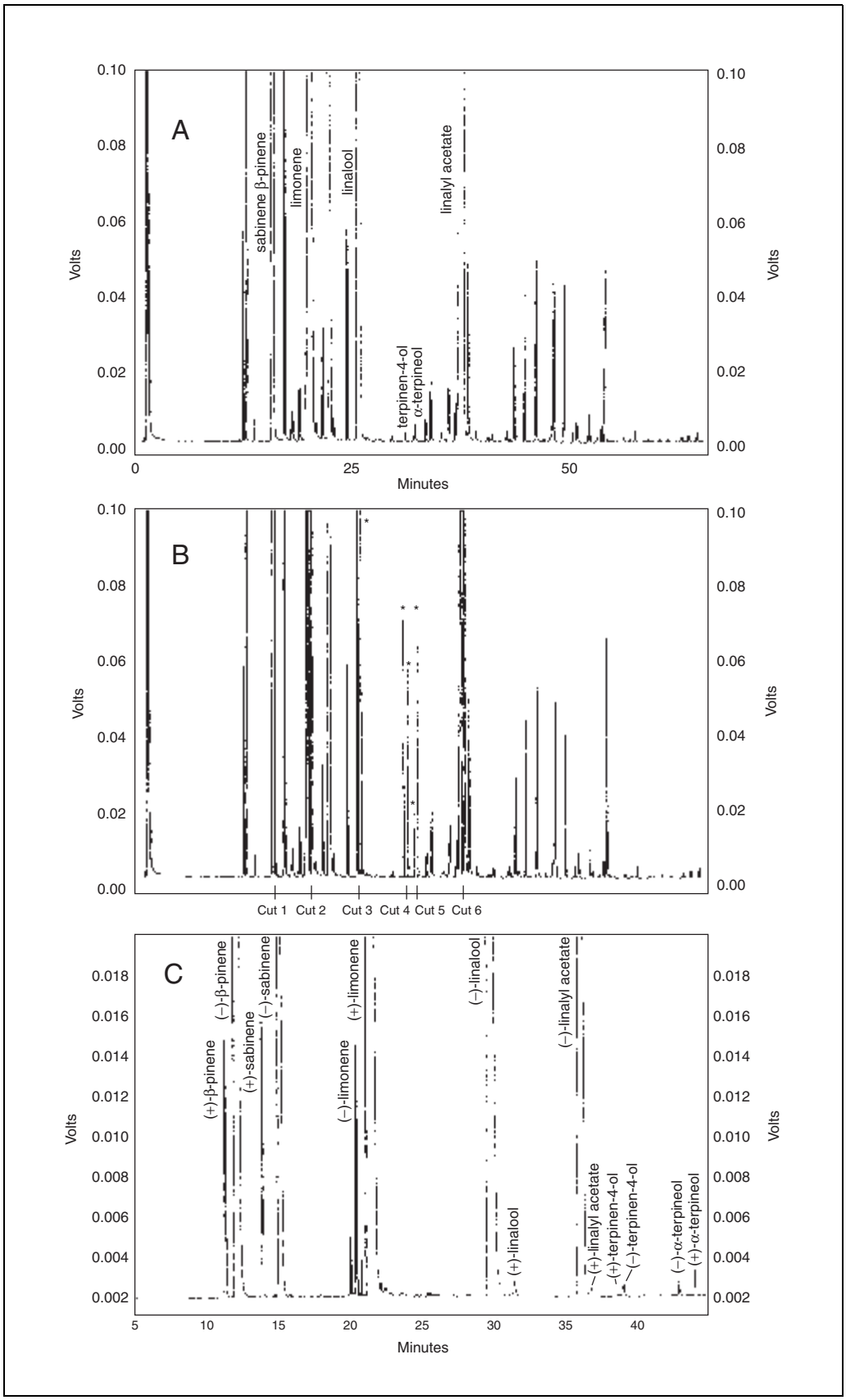

Figure G1.4.3 Chromatograms obtained using an MDGC system where 6 heart-cuts are made in one run. (**A**) Chromatogram obtained on an SE-52 precolumn, showing the peaks to be heart-cut. (**B**) The same sample showing where the cuts occurred. The asterisks are spikes caused by the valve switching. (**C**) The chromatogram obtained from the chiral analytical column showing the separation of all 6 components in the same run. Reprinted with permission from Mondello et al. Copyright 1998 American Chemical Society.

Sample preparation is similar to that of Basic Protocol 1. The precolumn can be viewed as the ideal technique for sample cleanup. No other sample cleanup is necessary. If the sample contains nonvolatile material, cleanup procedures 2 to 4 (see Basic Protocol 1) would help prolong the life of the precolumn, but it would not improve the quality of the separation on the analytical column.

Materials

Precolumn (achiral): poly(ethylene glycol) or a suitable polar stationary phase
Analytical column (chiral):
heptakis(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin in poly(14% cyanopropylphenyl/86% dimethylsiloxane)
C_7-C_{20} *n*-alkane hydrocarbon standard for a nonpolar precolumn, or C_9-C_{30} for a polar precolumn (hydrocarbon standard; see recipe)
Sample containing an on-column concentration of 20 to 80 ng of the enantiomers of interest
Liquid nitrogen

Multidimensional gas chromatograph with two linked independently operated ovens (first oven fitted with an on-column injector and both ovens fitted with FIDs)
Integrator for each detector or access to data collection software to record chromatograms for both detectors
10-µl syringes

Set operating conditions for an essential oil analysis or food extract

1. Set the following parameters:

Oven 1
Column: 30-m × 0.53-mm i.d. × 1.0-µm film thickness
Oven temperature: 30°C for 30 sec, 40°C/min to 60°C, 3°C/min to 200°C
Carrier gas: H_2 at a flow rate of 12 ml/min
Injector temperature: 150°C
FID detector temperature: 225°C
Sample size: 2 µl

Oven 2
Column: 30-m × 0.25-mm i.d. × 0.25-µm film thickness
Oven temperature: 40°C for 15 min, then 2°C/min
Carrier gas: H_2 at a linear velocity of 80 cm/sec
FID detector temperature: 225°C.

2. With oven 2 disabled by the switching valves or by using a separate instrument with the same column installed as the precolumn above and with the same operating conditions, carry out the following preliminary runs.

   a. Inject a 1-µl sample of the *n*-alkane hydrocarbon standard with a 10-µl syringe and record its chromatogram.

   b. Inject a 1-µl sample of the analyte with a 10-µl syringe and determine the time where each heart-cut will be taken from the chromatogram.

Use the dual oven instrument

3. Set all operating parameters and allow the instrument to stabilize for 30 min.

4. Enable the integrators, or software for data collection.

5. Set the time program for collecting the heart-cut fractions.

6. Turn on the liquid nitrogen valve to cool the trap, if it is not a part of the time program.
7. Inject a 2-µl sample of the analyte on to the precolumn with a 10-µl syringe and record its chromatogram showing the heart-cuts.
8. Record the retention time and peak areas of each heart-cut eluting from the analytical column.

9a. Calculate the percentage of each enantiomer present from Equation G1.4.3.

$$\text{enantiomeric purity } \% = \left[\frac{R}{R+S}\right]\times 100 \text{ and } \left[\frac{S}{R+S}\right]\times 100$$

where R and S represent the areas of the R and S peaks, respectively, of a pair of enantiomers in the chromatogram.

9b. Calculate the separation factor, α from Equation G1.4.4.

$$\alpha = \frac{t_{R2}}{t_{R1}}$$

where t_{R1} and t_{R2} are the corrected retention times for peak 1 and peak 2, respectively.

BASIC PROTOCOL 2

SENSORY DISCRIMINATION OF CHIRAL FLAVOR COMPOUNDS USING GAS CHROMATOGRAPHY-OLFACTOMETRY (GC-O)

Determination of the odor character and intensity of enantiomers relies heavily on complete separation of the components of the sample where there is no coelution and baseline separation of enantiomers is seen (see Fig. G1.4.4). If these ideal conditions are not met, considerable errors will be incurred in making odor measurements, particularly in cases where both enantiomers have similar odors, or where one is odorless. Traces of odorants coeluting with analytes under investigation, tailing of peaks, and low resolution all seriously affect chromatographic odor data. If the retention times of two enantiomers differ by <1 min, quantitative odor data may be inaccurate.

Sample preparation is similar to that of Basic Protocol 1. The sample cleanup procedures should ensure that no extraneous odorants are eluting with chiral odorants of interest. Thus, cleanup procedure 5 is preferred, which should be modified by attaching the polar column to the olfactometer port so that unwanted odorants can be detected. If odorant coelution is found, then a new chiral stationary phase must be chosen for the sensory analysis. Cleanup procedures 2 to 4 will prolong the life of the chiral column. Cleanup procedure 1 is given as Alternate Protocol 2 of this unit.

Materials

C_7-C_{20} *n*-alkane hydrocarbon standard for a nonpolar column, or C_9-C_{30} for a polar column (hydrocarbon standard; see recipe)

Sample

For qualitative evaluation: an on-column concentration of 20 to 80 ng of the enantiomers of interest

For quantitative evaluation: a set of 6 serial dilutions of the original sample that should have an accurately known concentration of ~2.0%. The concentration can be adjusted to give the optimum odor data. Each sample is diluted by a factor of 3 from the previous sample (i.e., 1 ml sample diluted with 2 ml of solvent) and then given a random number using a "double blind" labeling method. The samples are sniffed in random order.

High-resolution gas chromatograph fitted with the appropriate chiral column (e.g., heptakis(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin in poly(14% cyanopropylphenyl/86% dimethylsiloxane)), a stationary phase widely used for the chiral analysis of many food extracts

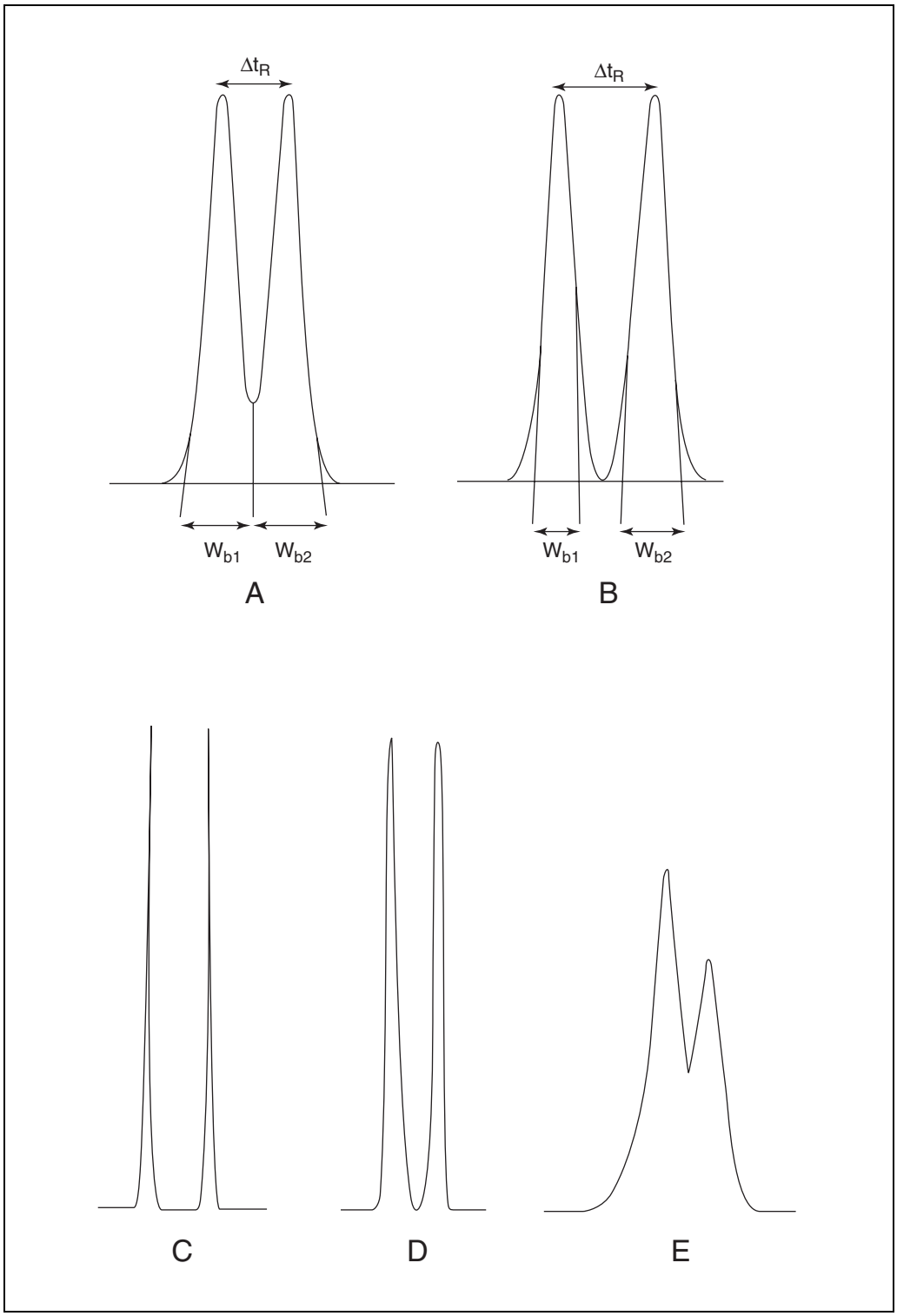

Figure G1.4.4 Resolution $R_s = 2\Delta t_R/(W_{b1} + W_{b2})$ at different separations. (**A**) sufficient separation, $R_s = 1$; (**B**) baseline separation, $R_s = 1.5$. C, D, and E have the same separation factor, but show problems cause by overloading the column. (**C**) Good resolution; (**D**) some peak broadening and tailing; (**E**) excessive tailing causing peak overlap.

FID detector installed in parallel with an olfactometer capable of delivering the effluent from the column to the human sniffer. The column is installed in either the FID port or the olfactometer port, depending upon the mode of operation. Split effluents are not recommended.
Integrator or access to data collection software to record chromatograms
Software package capable of recording timed events and odor descriptors such as Charmware (Datu)
10-µl syringe

Set operating conditions for an essential oil analysis or food extract

1. Set operating parameters:

   Column: 30-m × 0.32-mm i.d. × 0.25-µm film thickness
   Oven temperature: 40°C for 10 min, 2°C/min to 230°C, hold for 10 min
   Carrier gas: H_2 at a linear velocity of 80 cm/sec
   Injector temperature: 150°C
   Split ratio: 100:1
   FID temperature: 225°C
   Olfactometer block 225°C
   Temperature of the effluent reaching the sniffer: 35° to 40°C
   Sample size: 1 µl.

   Allow the instrument to stabilize for 30 min.

2. Enable the integrator, or software for data collection.
3. Inject 1 µl *n*-alkane hydrocarbon standard onto the column with the column in the FID port.
4. Record the chromatogram as a retention index standard for all runs performed under the same operating conditions (see Basic Protocol 1).
5. Move the column from the FID port to the olfactometer port and turn on the sniff air flows.

For qualitative analysis

6a. Inject a 1-µl sample of the sample onto the column and record the retention time and the descriptor for all odors eluting from the column by sniffing the effluent at the olfactometer port.

7a. Using the chromatogram obtained from Basic Protocol 1, operating condition 6 as a reference, identify the odors of enantiomers of interest by their retention index and determine whether contamination of odors has occurred caused by coelution or poor resolution.

8a. Assign descriptors to all resolved pairs of enantiomers where sensory discrimination has been detected.

For quantitative analysis

6b. Inject a 1-µl sample onto the column of a randomly chosen dilution from the set of serial dilutions, and record the retention time and odor descriptor for all odors eluting from the column by sniffing the effluent at the olfactometer port.

7b. Repeat step 6b until data has been collected for all 6 samples.

8b. Construct an odor chromatogram for the sample and use it to determine the odor activity value (OAV; Grosch, 1993) or the odor spectrum value (OSV; Acree, 1997) for the odorants of interest. From the odor spectrum identify the pairs of enantiomers and note their relative OSV's.

SENSORY DISCRIMINATION OF CHIRAL FLAVOR COMPOUNDS USING MULTIDIMENSIONAL GAS CHROMATOGRAPHY (MDGC)

ALTERNATE PROTOCOL 2

This method is used when accurate quantitative odor data are required and the separation produced by a single column gives overlapping odors that could not be separated by adjusting the operating conditions or reconfiguring the instrument. Traces of powerful odorants coeluting with enantiomers under analysis may cause extensive distortion of odor data and be present in concentrations that are not high enough to be detected by any detector except the sniffer. Changing the precolumn may solve one set of contamination problems, only to cause new ones. The basic instrument configuration is described in Alternate Protocol 1.

Sample preparation is similar to that of Basic Protocol 1. Once it has been established that no coelution of odorants is occurring with the chiral odorants of interest (see Basic Protocol 2), then the precolumn provides the only necessary sample cleanup. Only the heart cuts reach the analytical column.

Materials

Precolumn (achiral): poly(ethylene glycol) or a suitable polar stationary phase
Analytical column (chiral):
Heptakis(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin in poly(14% cyanopropylphenyl/86% dimethylsiloxane)
C_7-C_{20} *n*-alkane hydrocarbon standard for a nonpolar precolumn, or C_9-C_{30} for a polar precolumn (hydrocarbon standard; see recipe)
Sample for either qualitative or quantitative determinations (see Basic Protocol 2)
Liquid nitrogen

Multidimensional gas chromatograph with two linked independently operated ovens. The first oven is fitted with an on-column injector, both ovens are fitted with FIDs and the second oven has an olfactometer installed in parallel with the FID. The analytical column is installed in either the FID port or the olfactometer port as described (see Basic Protocol 2).
Integrator for each detector or access to data collection software to record chromatograms for both detectors
10-µl syringes

Set operating conditions for an essential oil analysis or food extract

1. Set operating parameters for each oven.

Oven 1
Column: 30-m × 0.53-mm i.d. × 1.0-µm film thickness
Oven temperature: 30°C for 30 sec, 40°C/min to 60°C, 3°C/min to 200°C
Carrier gas: H_2 at a flow rate of 12 ml/min
Injector temperature: 150°C
Detector temperature: 225°C
Sample size: 2 µl.

Oven 2
Column: 30-m × 0.25-mm i.d. × 0.25-µm film thickness
Oven temperature: 40°C for 15 min, then 2°C/min
Carrier gas: H_2 at a linear velocity of 80 cm/sec
Detector temperature: 225°C

2. With oven 2 disabled by the switching valves or by using a separate instrument with the same column installed as the precolumn and with the same operating conditions, carry out the following preliminary runs.

   a. Inject a 1-µl sample of an *n*-alkane hydrocarbon standard with a 10-µl syringe and record its chromatogram.

   b. Inject a 1-µl sample of the analyte with a 10-µl syringe and determine the time where each heart-cut will be taken from the chromatogram.

Use the two-oven instrument

3. Place the analytical column in the olfactometer port and turn on the sniff air flows.
4. Set all operating parameters and allow the instrument to stabilize for 30 min.
5. Enable the integrators, or software for data collection.
6. Set the time program for collecting the previously determined heart-cut fractions.
7. Turn on the liquid nitrogen valve to cool the trap if it is not a part of the time program.
8. Proceed as described in Basic Protocol 2, qualitative analysis steps 6a to 8a; quantitative analysis steps 6b to 8b.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Hydrocarbon standard

The hydrocarbon standard provides a universal scale (retention index, RI) for the characterization of volatile odorants. Since a single determination may require the use of more than one instrument (GC, GC-O, GC-MS), it is crucial that every time a run is made using new operating conditions, a new calibration is recorded using the hydrocarbon standard. This is the only way that RI data from one instrument can be compared with that from another provided that the stationary phase is the same. RIs do not vary with the operating conditions, while retention times do. (See more on the use of hydrocarbon standards in *UNIT G1.1*.)

The standard is composed of a series of *n*-alkanes, from C_7 to C_{30}. They should be dissolved in the same solvent that is used for dissolving the sample being analyzed. A suitable concentration is 100 µg/ml of each one, with two, e.g., C_{11} and C_{13}, at a concentration of 50 µg/ml to act as markers in the chromatogram. This stock solution should be stored at −10°C to minimize loss of low molecular weight alkanes and used as a stock solution. Dilute it by 1:10 for use as a standard. A convenient procedure for making up the stock solution follows.

Using a Pasteur pipet, determine the weight of 1 drop of *n*-decane. In a dark screw-top bottle, add 1 drop each of *n*-undecane and *n*-tridecane, 2 drops each of all other liquid alkanes, and the same weight of each of the solid alkanes. Record the weights of each one. Start by weighing the alkanes of highest molecular weight first to minimize loss by evaporation. Add the appropriate amount of pentane (or the required solvent) to make a solution of 100 µg/ml of each alkane in solution together. Store at −10°C.

continued

Working solution: dilute 1 ml of stock solution to 10 ml

For nonpolar columns use a standard composed of C_7-C_{18} *n*-alkanes

For polar columns use a standard composed of C_9-C_{30} *n*-alkanes

Over a period of time, preferential evaporation of the lower molecular weight alkanes will occur. Unless known weights of each alkane are required, this is not a serious problem. Using a working solution minimizes this problem and also avoids contamination. When the quality of the standard becomes unacceptable, a new sample can be diluted from the stock solution.

COMMENTARY

Background Information

Food scientists are interested in the enantiomer distribution of chiral food odorants because enantiomers may have different odors and odor intensities. Determination of enantiomer ratios and their sensory properties can provide information about origin of food aromas and the perceived variations in the taste of foods. These data can be collected only when the enantiomers are separated using enantioselective high-resolution gas chromatography, which is the leading method for stereodifferentiation of chiral food odorants.

More recently, enantiomer ratios have been used as evidence of adulteration in natural foods and essential oils. If the enantiomer distribution of a chiral component of a natural food does not agree with that of a questionable sample, then adulteration can be suspected. Chiral GC analysis alone may not provide adequate evidence of adulteration, so it is often used in conjunction with other instrumental methods to completely authenticate the source of a natural food. These methods include isotope ratio mass spectrometry (IRMS), which determines an overall $^{13}C/^{12}C$ ratio (Mosandl, 1995), and site-specific natural isotope fractionation measured by nuclear magnetic resonance spectroscopy (SNIF-NMR), which determines a $^{2}H/^{1}H$ ratio at different sites in a molecule (Martin et al., 1993), which have largely replaced more traditional analytical methods using GC, GC-MS, and HPLC.

The requirements for analyzing food odorants are demanding. The system must have a sensitivity of a few parts per billion or less, and be capable of handling highly volatile compounds. Although many methods exist for the analysis of chiral compounds, gas chromatography is the only viable method for analyzing food odorants because many are present in amounts too low for detection by most analytical methods. The development of gas chromatography-olfactometry has helped to improve the detection of odorants with low thresholds present in trace amounts in foods.

Until chiral stationary phases were developed, the separation of enantiomers using gas chromatography was tedious, and was of little value in flavor analysis. Currently, the stationary phases most often used are a wide range of modified α-, β-, and γ-cyclodextrins dissolved in polysiloxanes. They are quite versatile and many are thermally stable up to 230°C. Some are now available where the cyclodextrin is anchored to the polysiloxane by the method of derivatization, which increases their useful lifetime. OV-1701 and SE-54 with 10% to 30% modified cyclodextrin are two of the most versatile chiral stationary phases that are commercially available. There is no systematic way to determine the most suitable stationary phase for a particular separation because the role played by molecular recognition in the mechanism of chiral separation using cyclodextrins is not yet fully understood. The online database CHIRBASE is a collection of published separations, and may be consulted to find the best stationary phase for a specific application (CHIRBASE, 1992). The literature describing applications of chiral chromatography to flavor analysis and systematic selection of chiral stationary phases is disorganized. Several companies marketing cyclodextrin columns have excellent Web sites describing the capabilities of the columns they make, including flavor applications (Restek Corporation, 1997; Supelco, 1998; also see Internet Resources for ASTEC web site). There are over a hundred chiral columns that are commercially available. See Tables G1.4.1 and G1.4.2 for cyclodextrin columns that are readily available, together with some leading suppliers.

Important terms

1. Enantiomeric purity: the measured ratio % of the detected enantiomers.

$$\text{enantiomeric purity } \% = \left[\frac{R}{R+S}\right]\times 100$$

$$\text{and } \left[\frac{S}{R+S}\right]\times 100$$

where R and S represent the areas of the R and S peaks, respectively, of a pair of enantiomers in the chromatogram.

2. Enantiomeric excess or optical purity, ee, the relative difference of the separated enantiomers.

$$\%\text{ee} = \left[\frac{R-S}{R+S}\right]\times 100$$

where $R > S$.

3. Separation factor, α:

$$\alpha = \frac{t_{R2}}{t_{R1}}$$

where t_{R1} and t_{R2} are the corrected retention times for peak 1 and peak 2, respectively.

4. Peak resolution or separation efficiency, R_s

$$R_s = \frac{2\left(t_{R2} - t_{R1}\right)}{W_{b1} + W_{b2}}$$

where W_{b1} is the peak width at base of the less retained peak, and W_{b2} is the peak width at base of the more retained peak measured at 4σ, where σ is the standard deviation for the peak.

5. Retention time, t'_R

$$t'_R = t_R - t_M$$

where t_R is the total retention time; time (or distance) from the point of injection to the point of peak maximum and t_M is the gas hold-up time; minimum time (or distance) required for the elution of a nonretained substance.

6. Retention index, RI

$$\text{RI}_i = 100n + 100\left[\frac{\log x_i - \log x_n}{\log x_{n+1} - \log x_n}\right]$$

where x_n is the corrected retention time of the n-alkane eluting before x_i.

7. Capacity ratio (partition ratio), k

$$k = \frac{t'_R}{t_M}$$

Table G1.4.1 Commercially Available Cyclodextrin Columns

2,3,6-Tri-*O*-methyl-β-cyclodextrin in OV1701[a]
2,3,6-Tri-*O*-methyl-γ-cyclodextrin in OV1701[a]
2,3,6-Tri-*O*-pentyl β-cyclodextrin[a]
2,3,6-Tri-*O*-pentyl-α-cyclodextrin
2,6-Di-*O*-pentyl-3-*O*-butyryl-γ-cyclodextrin[a]
2,6-Di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin in OV1701[a]
2,6-Di-*O*-pentyl-3-trifluoroacetyl α-cyclodextrin
2,6-Di-*O*-pentyl-3-trifluoroacetyl β-cyclodextrin
2,6-Di-*O*-pentyl-3-trifluoroacetyl γ-cyclodextrin[a]
2,3-Di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl-β-cyclodextrin
2,3-Di-*O*-ethyl-6-*O*-*t*-butyldimethylsilyl-β-cyclodextrin
2,3-Di-*O*-ethyl-6-*O*-*t*-butylsilyl-β-cyclodextrin
2,3-Di-*O*-acetyl-6-*O*-*t*-butyldimethylsilyl-β-cyclodextrin in OV1701[a]
2,3-Di-*O*-acetyl-6-*O*-*t*-butyldimethylsilyl-γ-cyclodextrin in OV1701[a]
O-(*S*)-2′-Hydroxypropyl-per-*O*-methyl α-cyclodextrin
O-(*S*)-2′-Hydroxypropyl-per-*O*-methyl β-cyclodextrin[a]
O-(*S*)-2′-Hydroxypropyl-per-*O*-methyl γ-cyclodextrin
2,6-Di-*O*-pentyl α-cyclodextrin
2,6-Di-*O*-pentyl β-cyclodextrin
2,6-Di-*O*-pentyl γ-cyclodextrin
2,6-Di-*O*-pentyl-3-*O*-acetyl α-cyclodextrin
3-*O*-Acetyl-2,6-Di-*O*-pentyl β-cyclodextrin
3-*O*-Butyryl-2,6-Di-*O*-pentyl γ-cyclodextrin 60% in OV 1701

[a]Widely used columns.

8. Odor activity value OAV

$$OAV = \frac{\text{concentration of odorant}}{\text{detection threshold in sample}}$$

9. Odor spectrum value OSV. Normalized odor data taken from each peak of the chromatogram that has been adjusted for odor compression using Stephen's Law (Acree, 1997; Ong et al., 1998). The odor chromatogram is redrawn as an odor spectrum. An odor spectrum value can be determined for each odorant in the sample. The values are independent of the method used for collecting odor data and of the concentration, and convey the relative importance of each odorant in the sample.

The measurement of odor intensity using OAVs is described by Grosch (1993, 1994). It requires the determination of the concentration of each odorant in the sample, and for those present in trace quantities, a stable isotope dilution assay must be used (Guth, 1997). This may make the determination of OAVs very tedious if many values are required. OSVs are normalized peak areas from an odor chromatogram and represent a more realistic representation of the importance of the odors in a sample as perceived by the nose. Their determination is described by Acree (1997).

Critical Parameters

When reliable chiral data about food odorants are required, it is often important to have good information about the nature and identity of the compounds under analysis before a chiral analysis is attempted. The information below may be essential for a good analysis, and methods for obtaining these data are described elsewhere.

1. Identity of odorants to be analyzed, determined by GC-MS.

2. A guarantee that the enantiomers are stable under the conditions of the analysis, and that no racemization occurs.

3. The order of elution of the *R* and *S* isomers, determined by comparison with a known authentic sample analyzed under the same conditions as the unknown.

4. Concentration of odorants in sample being analyzed, determined by use of internal standards, GC-MS in selected ion monitoring (SIM) mode or a stable isotope dilution assay for trace quantities (see *UNIT G1.3*).

5. Retention index of odorants on the stationary phase used in the chiral analysis (single oven) or on the precolumn (MDGC), and on more than one stationary phase if found to be necessary for the identification of odorants, determined by using a hydrocarbon standard, described above and in *UNIT G1.1.*

6. Odor descriptors of odorants to be analyzed and of those coeluting with analytes, determined by GC-O analysis. It may be important to know the odor descriptors for pairs of enantiomers in cases where contamination occurs. It may also be important in cases where one enantiomer is odorless, or where both enantiomers have similar odors but different intensities.

7. A sample that has been concentrated by preparative GC or MDGC to enable 1 and 4 to 6 described above to be determined. Concentration techniques are described in *UNIT G1.2*.

Table G1.4.2 Suppliers of Chiral Capillary Chromatography Columns

Advanced Separation Technologies ASTEC
Alltech Associates
Carlo Erba Reagenti
Chrompack International
Chrom Tech
CS-Chromatographie Service
J & W Scientific/Fisons
Macherey-Nagel GmbH
Mega
Restek
SGE International
Sumitomo Chemical
Supelco
Technicol
TPC Ziemer

Selection of instrumentation

The success of a chiral analysis will ultimately depend upon the instrumentation available. Single dimensional separations have become routine with a large selection of efficient chiral columns now available (see Table G4.1.1), but they have some limitations. Many powerful odorants are present in trace quantities, and are often hidden in the chromatogram by coeluting compounds, and may not show in the chromatogram, even if they can be made to elute alone. A one-oven system will provide reliable data when enantiomers are not coeluting, and the sensory data can be assumed to be for a single compound. If it is believed that coelution is occurring, a simple method to confirm that suspicion is to obtain a new chromatogram using a different stationary phase. If this is followed by a GC-O run using the new column, then the integrity of the odor data can be established. The Flavornet database (Acree and Arn, 1997), a developing source for the identification of flavor compounds by GC-O, is very useful for tracking flavor compounds by their retention indices on different stationary phases.

If more efficient chromatography is needed for improved separations and reliable quantitative data, then a multidimensional system must be used. The analysis can hardly be described as routine, since the cost of commercially available instrumentation is high, and there are very few instruments that are currently available (see Table G1.4.3). Many reported enantiospecific analyses described in the literature use an instrument that is either no longer in production (Mosandl et al., 1990; Bernreuther and Schreier, 1991) or is a sophisticated custom-built system (Mondello et al., 1999). Several designs that have been used for the switching device that transfers small portions of the first chromatogram to the second column have been described (Wright, 1997; Bertsch, 1999). The challenges confronting the flavor chemist when accurate enantiospecific data are required are described in the analysis, identification, and quantification of the sulfur odorants of passion fruit (Werkoff et al., 1998).

MDGC performed using a single oven is not a preferred configuration for the analysis of complex mixtures of food volatiles. It is important to keep in mind that no chromatographic system has yet been devised that is capable of completely resolving all the components of a complex mixture.

Selection of operating conditions

Use of the literature is strongly recommended as the starting point for determining the optimum operating conditions for a separation. The ultimate goal is for the separation factor α to be as large as possible. The chiral stationary phase, temperature program, gas flow though the column, and sample size are key parameters for a successful separation (König, 1992; Sponsler and Biederman, 1997).

Chiral columns are often operated isothermally at temperatures between 40° and 60°C, or with heating rates significantly lower than for routine analyses.

On-column injection is preferred, because it occurs at room temperature. Hot injector ports can lead to decomposition of the sample, and to racemization of chiral components. If a single oven system is used, then split injection offers some advantages because it is important not to expose the chiral column to large amounts of solvents. With an MDGC system, the heart-cutting technique removes the solvent from the chiral column, so on-column injection is preferred.

Hydrogen is the preferred carrier gas because runs are shorter and the resolution is superior. Helium will give satisfactory results unless α is close to 1.0. Linear velocities using hydrogen are usually ~80 cm/sec, which is higher than for routine separations because it results in narrower peaks.

Sample size must be kept to a minimum, 40 to 80 ng on-column concentration for chiral components, otherwise overloading, broadening of peaks, and loss of resolution will occur.

Selection and care of chiral columns

Column selection is not yet a precise science. There are no uniform theories for the separation mechanisms that operate for the resolution of enantiomers using the wide array of chiral stationary phases that are currently available. It is therefore strongly recommended that the literature is searched to determine whether the analyte under investigation has been separated and which chiral stationary phase was used. Many chiral food volatiles have now been separated and a comprehensive database has been compiled that is continually updated and contains most reported separations (Koppenhoefer et al., 1993; Roussel and Piras, 1993). There are many other sources in the literature (Anonymous, 1993a,b; König, 1993; Maas et al., 1994; Schreier et al., 1995; Juchelka et al., 1998; Miranda et al., 1998).

Modified cyclodextrins are the most versatile and widely used chiral stationary phases. The most widely used columns contain 10% to 50% cyclodextrin dissolved in either OV-101 or SE-52 polysiloxane. They are thermally stable up to 230°C but require some care in use because cyclodextrins are soluble in many solvents and can be washed off the column if they are exposed to too much solvent.

Considerations in selecting a column

To select a chiral column, first the literature should be consulted, and then the availability of the column should be assessed (see Tables G1.4.1 and G1.4.2).

Use standard lengths (25 to 30 m). To avoid overloading the column, the capacity can be increased by using a thicker film thickness and a wider diameter. This will not alter the separation efficiency. Occasionally, very short columns (2 m) give better results.

Care of CD columns

New columns should be conditioned following the manufacturer's instructions. Most are conditioned at lower temperatures than for achiral columns. The temperatures used for separation should be as low as possible, which leads to better separation and less deterioration of the sample.

The column should not be exposed to unnecessarily large volumes of solvent. Columns can be stored in a clean dry atmosphere, but they should be sealed by a flame or taped for very short periods of time. Some columns need to be stored in an atmosphere of nitrogen to avoid serious loss of selectivity. The manufacturer's instructions should be followed for column storage.

Troubleshooting

Most analytical problems arise from poor separations and coelution of analytes. Some selected problems are discussed.

If the separation factor approaches 1.0, possible causes include: chiral stationary phase is incorrect, carrier gas velocity is too high, or only one enantiomer is present in the sample.

If the separation is acceptable but the resolution is poor (see Fig. G1.4.2), possible causes: the temperature program is incorrect, carrier gas velocity is incorrect, column is overloaded, focussing of the heart-cut sample is insufficient (MDGC), the column is old and has lost efficiency, or chiral stationary phase is inappropriate.

If coelution is occurring, possible causes: an incorrect polysiloxane is in the chiral column, the temperature program is wrong, the effect on different components may vary, MDGC may be needed, or an unsuitable precolumn is used. It is easier to solve coelution problems on the precolumn than on the analytical column.

The sensory data are inconsistent and coelution is suspected. If the solutions given above fail, then the problem may be difficult to resolve because the contamination may be occurring from an odorant that is present in such small quantities that it is only detectable by GC-O. Possible causes: the separation factor α is too small, tailing of peaks is occurring, both enantiomers have the same odor, or one enantiomer is odorless. Interaction of coeluting odors may vary with small variations in the concentrations of any of the odorants; the note of an odorant can vary with concentration.

Table G1.4.3 Instrumentation: Multidimensional Gas Chromatography Systems

Currently available	
Microanalytics Instrument	Complete MDGC systems are available, suitable for chiral aroma analysis
SGE International	The switching device is sold as an accessory to attach to a single or dual oven system
Systems widely used that are no longer in production	
Siemens AG	Sichromat 2 double-oven system with a "live switching" coupling piece and independently controlled ovens
Chrompack International	Multiple Switching Intelligent Controller (MUSIC) system, based on the Deans pressure switching concept
Custom-built systems	Many are described in the literature. An example of a sophisticated system can be found in Mondello et al. (1999)

Alternative methods for overcoming problems caused by coelution and poor resolution are as follows:

1. Synthesize chiral compounds of interest and determine OSVs of enantiomeric pairs as pure authentic samples.
2. Use a trained sensory panel to collect data when separation factors approach 1.0. The odor data can then be analyzed using statistical methods.
3. Using a different achiral stationary phase will alter the order of elution. This may eliminate some coelution problems, but new ones may occur. It may alter the order of elution of the *R* and *S* isomers.

Anticipated Results

Enantiomer ratio

Values for the enantiomer ratio found in natural products can range from 0% to 100%. If the enantiomeric purity is 100%, care must be taken to determine which isomer is present by comparison of retention times with a known standard. Samples from different geographic or growing regions may show some variation in the ratio of a specific compound, while others will not deviate from a known value.

Knowledge of enantiomer ratios, particularly for compounds that occur naturally with 100% of one enantiomer, can form the basis of an authenticity determination (Kreis and Mosandl, 1992; Mosandl and Juchelka, 1997). In such analyses, deviations from known enantiomer ratios of authentic samples can indicate adulteration of the sample, where synthetic materials have been added, or the sample has been distilled although it was labeled as a natural product.

Limit of detection. A compound must give an FID response to be detected and quantified. This requires a concentration of a few ppm if the resolution is high and coelution is not occurring. Using SIM (see *UNIT G1.3*), odorants can be quantified at the ppb level.

Enantiospecific sensory evaluation

The integrity of the sensory data will depend upon the enantiomers being of the highest chemical and optical purity. This requires odorants to elute as single compounds without tailing of peaks with at least 30 to 60 sec between each enantiomer. These conditions may be unattainable since sensory measurements are very susceptible to the presence of trace impurities of other odorants.

If qualitative data are required, and the enantiomers differ significantly in their odor quality, then acceptable results may be obtained. If odor intensity measurements (OAVs or OSVs) or threshold values are required, then the conditions described above must be obtained if the data is to be of value. Bernreuther et al. (1997) and Koppenhoefer et al. (1994) have published the enantiospecific sensory data for a variety of chiral odorants.

Limit of detection. Any odorant present above its threshold level can be detected in a GC-O experiment. This can vary from a few ppb to a ppt level or higher. Odorants present in trace quantities are frequently below the limit of detection of an FID, so GC-O is the only method available for their identification. GC-O coupled with SIM provides the most sensitive method of identifying and quantifying trace odorants.

Time Considerations

Once the optimum operating conditions are established, individual runs can be quite short, depending upon the retention time at which enantiomers elute, from 10 to 100 min. Most enantiomers of interest can be made to elute in <30 min when a single oven system is used. The instrument has to be cooled and stabilized before another run can be carried out, so the total time from one run to the next will be ~1 hr. The run time will depend mostly upon the temperature program.

For an MDGC system, the total run time will depend upon the instrument configuration, and on how the heart-cut fraction is transferred from oven 1 to oven 2. If each enantiomer is examined separately on the analytical column, then the total run make take only 10 to 15 min longer than the time for a single oven system. However, this will require a run for each enantiomer examined. If all the heart-cuts in a sample are examined in a single run on the analytical column, then a run will take 2 to 3 times as long as the time for a single oven system and it will depend upon the temperature program for the analytical column. Heating rates are rarely >2°C/min.

On a 30-m nonpolar column, the chromatogram for a hydrocarbon standard C_7 to C_{20} with a temperature program of 2°C/min starting at 35°C will take 90 min to run with an alkane eluting every 6 to 8 min. With a rate of 6°C/min, the run will take 40 min, with an alkane eluting every 2 to 3 min.

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Analysis of Citrus Oils

The recovery of citrus oils goes back hundreds of years to the sale of lemon oil in Sicily. Citrus oil is recovered as cold-pressed oil, essence oil, or *d*-limonene. The term cold-pressed oil refers to citrus oil recovered during juice extraction, whereas essence oil is captured during evaporation. Citrus oils are composed of 90% monoterpenes, with orange and grapefruit oils containing 95% *d*-limonene. The quality of citrus oils determines their function and market value. Most cold-pressed oils are concentrated (folded) and then fractionated. The fractionated oil is either sold or used as a flavor precursor. Mandarin, lime, and lemon oil are typically processed for their oil and sold to flavor houses. Other oils are added back to juices to enhance flavor. Identification of volatile compounds is determined by chemical and physical parameters. The following protocols are designed to measure total available oil and to evaluate quality and composition of citrus oils. Gas chromatography characterizes the differences between cultivars, while titration techniques quantify *d*-limonene, aldehyde, and ester content.

STRATEGIC PLANNING

The following protocols are primarily conducted to ensure the quality of citrus oils during various phases of citrus processing. Citrus processors must account for quality and quantity of citrus oils sold to flavor houses. This not only includes monitoring recovered oils (i.e., cold-pressed oil, essence oil, and *d*-limonene) but also the total available oil.

Basic Protocols 1 and 2 provide flavor profiles for orange, mandarin, grapefruit, lemon, and lime oil. These profiles determine price and function depending on the composition and are gas chromatography (GC) techniques based on 30- to 45-min runs. However, verification of compounds at specific retention times is time consuming, with both the column and the flow rate effecting retention times. In addition, quantification can be a lengthy process. The generation of standard curves for quantitative analysis requires multiple runs of individual components. However, time should be minimized once verification and standard curves are established for individual compounds, provided GC conditions remain constant. Basic Protocol 3 is another GC technique researchers can use to confirm the purity of *d*-limonene. This is a 30-min assay based on an FID response.

In addition to chemical assays, Basic Protocols 4 through 6 evaluate the physical properties of citrus oils. These methods are based on the high percentage of *d*-limonene, in which citrus oils have characteristic values. Table G1.5.7 provides the physical and chemical properties defined by Food Chemical Codex (NRC, 1981).

Basic Protocol 7 applies more directly to citrus processors. It measures the total available oil. These procedures are also based on the level of *d*-limonene (>95%) in the sample. According to EPA regulation, processors must account for both recovered oil and emission vapors (*d*-limonene). The regulation permits 100 tons of volatile organic compounds per year. This is a highly accurate titration method with results slightly skewed for lemon and lime samples due to the various terpenes. However, most lemon- and lime-processing plants recover a higher percent of the total available oil and are in compliance with EPA regulations. Alternate Protocols 1 to 3 measure oils in press cakes, dry peel, and press liquor.

Basic Protocols 8 and 9 look at specific groups of compounds, aldehydes and esters, which are of more interest to flavor houses and research scientists. For flavorists, the strength of "citrus flavor" is determined from the aldehyde content, while the "fruity aroma" is attributed to the ester content. Over the years, these procedures have been modified. Basic Protocol 8 is a titration method to determine aldehyde content in citrus oils. It was originally developed for lemon oil; however, it is also applicable to other citrus oils. Alternate Protocols 4 and 5 also produce similar results. Alternate Protocol 4 is based on

Contributed by Trevor Gentry
Current Protocols in Food Analytical Chemistry (2002) G1.5.1-G1.5.24

citral levels, the predominate aldehyde in lemon oil. Alternate Protocol 5 is a spectrophotometric method, in which additional equipment and reagents are needed. Basic Protocol 9 is also a colormetric assay for determining ester content in citrus oils. It is a rapid technique with accurate results.

Basic Protocols 3 through 9 are primarily useful as quality control measures. They are rapid, usually within 30 min, given reagent preparation. The results are used to monitor the quality of a process. These results support established values for "high" quality citrus oil. Basic Protocols 1 and 2 are more involved and are better suited for research purposes. The equipment is more sensitive and also more expensive. Furthermore, the strength of the GC analysis can be enhanced by the addition of a mass spectrometer to identify either contaminates or unknown compounds present in a sample.

BASIC PROTOCOL 1

QUALITATIVE ANALYSIS OF CITRUS OILS BY GAS CHROMATOGRAPHY

The desired citrus flavor is the result of volatile compounds in specific proportions. Citrus oils have unique composition profiles depending on the cultivar, the processing conditions, and the storage conditions. There have been more than 200 different compounds identified in citrus oils. However, the degree of unsaturation in monoterpenes leads to rapid oxidation and unstable compounds. The reactivity and volatility of citrus oils require strict quality control protocols.

The quality of citrus oils is based on the purity of the sample as determined by a gas chromatogram (GC) profile. A small sample is injected onto a column, which separates the individual components. Separation is based on the physical interaction between the column and the sample as indicated by retention times. Figure G1.5.1 illustrates the GC setup.

Materials

Acetone
Citrus oil

Gas chromatograph including:
Flame ionization detector (FID)
Integrator
Nonpolar (DB-1, SE-30, OV-1) or weakly polar (DB-5, SE-52, Rt-5) column: 20 m × 0.10-mm i.d. with 0.1-µm film (J & W Scientific)
10-µl syringe

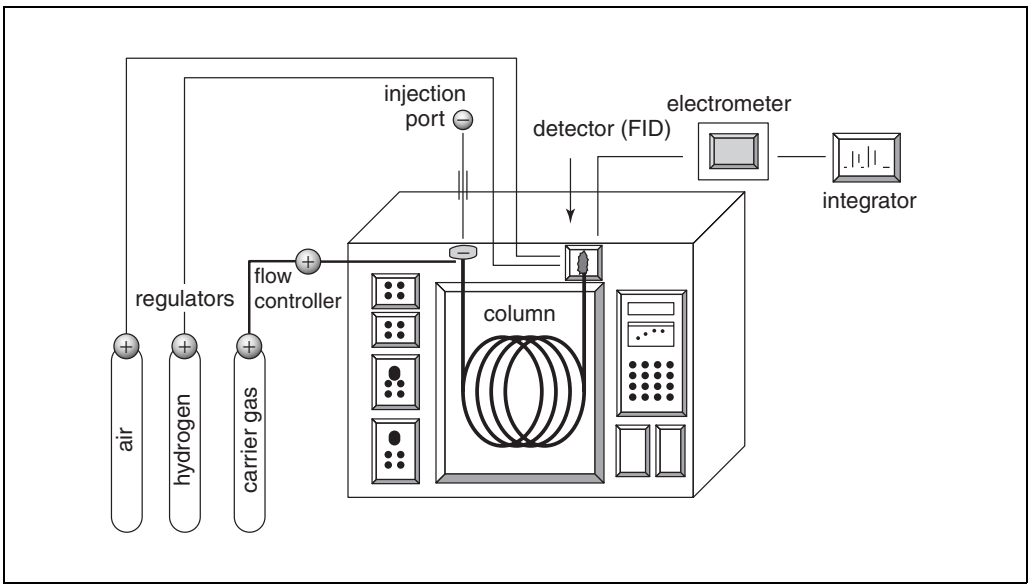

Figure G1.5.1 Schematic diagram depicting a gas chromatography setup.

1. Set up GC with the following conditions and ignite FID.

   Injector: 275°C
   Detector: FID 350°C, nitrogen make-up gas (30 ml/min)
   Carrier gas: 60 cm/sec hydrogen, split ratio 1:275.

2. Input the following temperature program for the oven:

   70°C for 1 min
   heat to 250° at 30°C/min
   heat to 310° at 20°C/min
   hold at 310°C for 2 min
   cool to 40°C.

3. Rinse 10-µl syringe 2 to 3 times with acetone.
4. Rinse 10-µl syringe 2 to 3 times with citrus oil sample.
5. Press start and inject 1.0 µl citrus oil sample.
6. Replicate samples as needed.

QUANTITATIVE ANALYSIS OF CITRUS COMPOUNDS BY GAS CHROMATOGRAPHY

BASIC PROTOCOL 2

Gas chromatography provides a rapid analysis of citrus oil quality. This technique can be further enhanced to determine quantitative levels of individual compounds. Compounds can be measured based on the FID response. A standard curve with known concentrations is used to extrapolate an unknown concentration.

Materials

Sample to be analyzed
Standard (compound of interest)
Solvent (e.g., ethanol, hexane)
100-ml volumetric flask

Additional reagents and equipment for gas chromatography (see Basic Protocol 1)

1. Perform GC analysis of the sample as described above (see Basic Protocol 1).
2. Weigh 0.1 mg standard into a 100-ml volumetric flask, dissolve in an appropriate solvent, and bring volume to 100 ml (final 10 ppm).

   Check the solubility of the compound in solvent prior to the experiment.

3. Prepare a series of concentrations down to 0.1 ppm following the dilution scheme in Figure G1.5.2.

   This scheme generates seven standards covering a range of 0.1 to 10 ppm, which will cover the concentration of most compounds. Data must be within the linear region of the curve.

4. Press start and inject 1.0 µl of the first standard onto the column. Record response. Perform analysis of each concentration three to four times.

   Standards should be quantified in order of increasing concentration, to prevent carry-over responses.

5. Average the replicate responses at each concentration and then generate a standard curve by plotting response versus concentration.

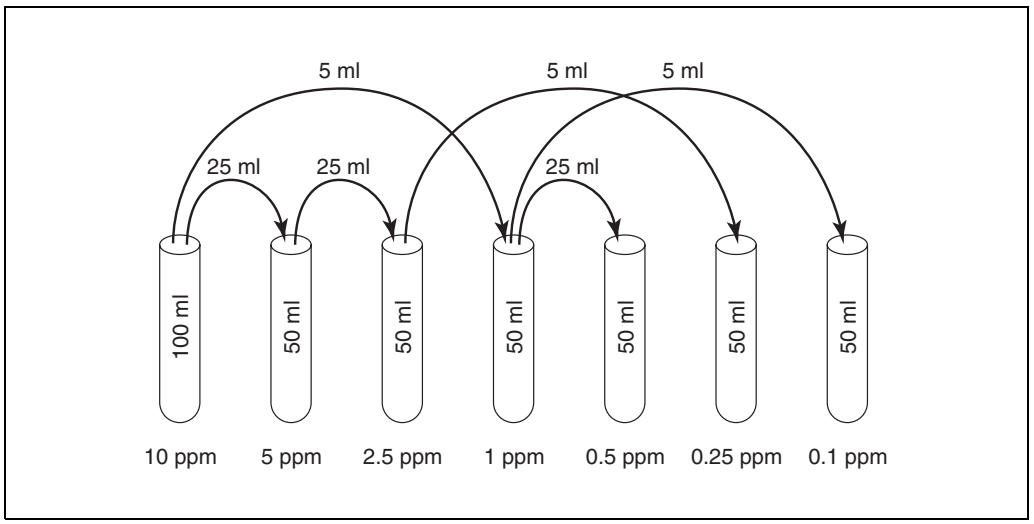

Figure G1.5.2 Dilution scheme for quantifying compounds.

6. Calculate the concentration of the unknown sample using the equation:

   [unknown] = (response − intercept)/slope

   This equation is derived from the basic linear regression equation, y = mx + b, where y is response, m is slope, x is concentration of the unknown, and b is the y intercept.

BASIC PROTOCOL 3

ANALYSIS OF LIMONENE PURITY BY RAPID GAS CHROMATOGRAPHY

Limonene is the major monoterpene in orange oil. This is a colorless and odorless compound at high purity. However, it rapidly oxidizes to carveol and carvone in the presence of air. Under acidic conditions, α-terpineol, β-terpineol, and γ-terpineol are also produced. Many of the impurities present in limonene have much higher odor potencies. These odor potent compounds can be perceived as "limonene odor".

Basic Protocol 3 is a rapid GC method to evaluate limonene purity. The needle is coated with a residual limonene layer in the syringe. The sample volatilizes into the column in which the sensitivity of GC detectors (FID) provides a reliable indication of purity. This is known as a "wet needle" injection.

Materials

Limonene sample
Additional reagents and equipment for gas chromatography (see Basic Protocol 1)

1. Follow the same GC/FID conditions for citrus oil analysis (see Basic Protocol 1).
2. Rinse needle with limonene sample.
3. Withdraw and then discard 1.0 µl of sample.

   This "wets" the needle, creating a limonene residue on the inside surface.

4. Press start and inject needle into septum.
5. Withdraw the plunger slightly (1 to 2 µl) and rapidly depress plunger.

   The combination of the pressure created from the plunger and temperature of the injection port will cause the sample to volatilize and enter the gas phase.

6. Determine limonene purity as the % peak area for the limonene response compared to the total area of the chromatogram.

ANALYSIS OF CITRUS OILS BY PYCNOMETRY

BASIC PROTOCOL 4

Specific gravity is the density (mass per volume) of the sample compared to the density of water (see Table G1.5.7 in Anticipated Results). Samples are equilibrated to the same temperature and weighed using a pycnometer. The pycnometer is a sample bottle that provides a constant volume.

Materials

Citrus oil, 25°C
25°C water bath
Pycnometer (VWR Scientific Products)
Analytical balance (Fisher)

1. Fill pycnometer with citrus oil cooled to 25°C.

   A specific gravity vial (VWR Scientific Products) can be substituted for a pycnometer.

2. Place pycnometer in a 25°C water bath and adjust citrus oil level as needed.

   Oils will expand or contract according to temperature.

3. Put cap on pycnometer and dry.
4. Weigh on an analytical balance and record.
5. Repeat procedure using the same pycnometer with water at 25°C.
6. Calculate the specific gravity (SG) using the equation:

   SG = oil weight/water weight

ANALYSIS OF CITRUS OILS BY REFRACTIVE INDEX

BASIC PROTOCOL 5

The refractive index is another physical property used to identify citrus oils. A refractometer is based on the law of refraction, or Snell's law, where the angle of refraction is a function of the sample. The sample is placed between two prisms. The light is trapped within the lower material (oil) and is completely reflected internally at the boundary surface. The field is then adjusted until the light and dark sections are equal. The line separating the two halves is known as the critical ray. The critical ray falls along a fixed scale. The instrument readings are calibrated to known samples (i.e., water or glass) with automatic corrections for temperature. The reliability of this procedure correlates to the high limonene content (>90%) in citrus oils.

Materials

Citrus oil
Refractometer

1. Record temperature of citrus oil.
2. Place 1 to 2 drops citrus oil on the glass prism of a refractometer.
3. Adjust the line between light and dark fields until distinct.
4. The readings are indexes of refraction. These readings are a conformation of the standard of identity for a citrus oil. The composition of these oils provides a precise reading.

   Most modern refractometers have built-in temperature corrections.

BASIC PROTOCOL 6

ANALYSIS OF CITRUS OILS BY POLARIMETRY

Optical rotation measures the degree that light is rotated (see Table G1.5.7 in Anticipated Results). In citrus oils, *d*-limonene is the major enantiomer in the sample. Since other optically active compounds are often present in racemic mixtures, there is no net rotation and thus they are ignored. If a compound is a racemic mixture, the polarimeter will not give a reading. Readings can be verified with known standards.

Materials

Citrus oil
Polarimeter with 25-mm polarimeter tube (VWR Scientific Products)

1. Adjust citrus oil to 25°C.
2. Place 25-mm polarimeter tube in trough of polarimeter between polarizer and analyzer.
3. Adjust analyzer until both halves have equal light intensities.
4. Determine direction of rotation based on this adjustment.

   The direction is positive (+) if the analyzer was adjusted clockwise, negative (−) if adjusted counterclockwise.

5. Read degrees directly. Multiply number by 4 (to normalize to a 100-mm tube standard).

BASIC PROTOCOL 7

QUANTIFICATION OF TOTAL OIL FROM WHOLE FRUIT OR WET PEEL

The processing of citrus oil is highly regulated. Processors must account for total available oil, recovered oil, and emitted oil. This is done primarily for environmental reasons, due to the reactive nature of terpenes. The total available oil varies depending on the cultivar.

The amount of oil in a sample is determined by Scott oil analysis (AOAC, 1990e). This is a bromination reaction previously used to determine the number of fatty acid double bonds. This titration method quantifies the recoverable oil in fruits and fruit products based on the release of Br_2 and the formation of limonene tetrabromide (Braddock, 1999). Figure G1.5.3 illustrates the chemical reaction for the bromination of limonene. Other monoterpenes (α-pinene and citral) also react; however, the method is accurate to within 10 ppm limonene (Scott and Valdhuis, 1966). For this procedure, limonene is co-distilled with isopropanol and titrated with a potassium bromide/bromate solution.

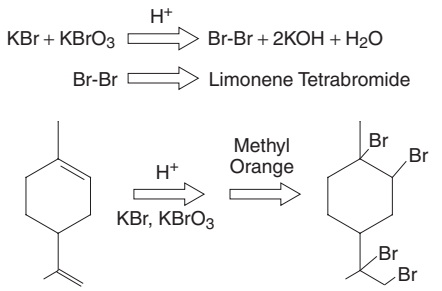

Figure G1.5.3 Chemical reaction of Scott oil analysis. Reprinted from Braddock (1999) with permission from John Wiley & Sons, Inc.

Materials

Whole fruit or wet peel (shredded)
Isopropanol
Methyl orange indicator solution: 0.5% (v/v) methyl orange in 1:2 (v/v) HCl
0.1 N $KBr/KBrO_3$ solution (see recipe)

3-liter blender (e.g., Waring Blendor)
Distillation apparatus (Fig. G1.5.4): 500 ml round-bottom flask with 24/40 neck attached to an adapter with a 28/15 ball connected to a 250-ml flask
Glass beads
Electric heater
25-ml buret

Additional reagents and equipment for determining moisture content (see Support Protocol 1)

1. Determine moisture content of sample (see Support Protocol 1)
2. Quarter whole fruit. Weigh 500 g sample (whole fruit or wet peel) into a 3-liter blender. Add 2500 g distilled water. Mix 2 min at medium speed and 1 min at high speed.
3. Place 25 g homogenate into a distillation flask with three to four glass beads.

   Samples should be analyzed in triplicate.
4. Add 25 ml isopropanol to homogenate.
5. Begin distillation and collect 25 to 30 ml distillate.

   Figure G1.5.5 shows the distillation set-up.
6. Add 10 ml methyl orange indicator solution to distillate.
7. Titrate with 0.1 N $KBr/KBrO_3$ solution to an endpoint of pH 5.4.
8. Calculate the total oil from whole fruit or peel using the equation:

   g oil/g peel = (x ml 0.1 N titrant/25 g sample) × (3000 g homogenate/500 g peel) × (0.004 ml oil/ml titrant) × (0.84 g oil/ml oil)

   The 0.004 ml oil/ml titrant is a correction factor based on the standardization of the potassium bromide/bromate solution.
9. Convert to lb. oil/ton dry solids using the equation:

   lb. oil/ton dry solids = (lb. oil/lb. peel) × (2000 lb. peel/ton peel) × (y ton peel/ton dry solids)

   where y ton peel/ton dry solids is the inverse of % solids, measured in step 1.

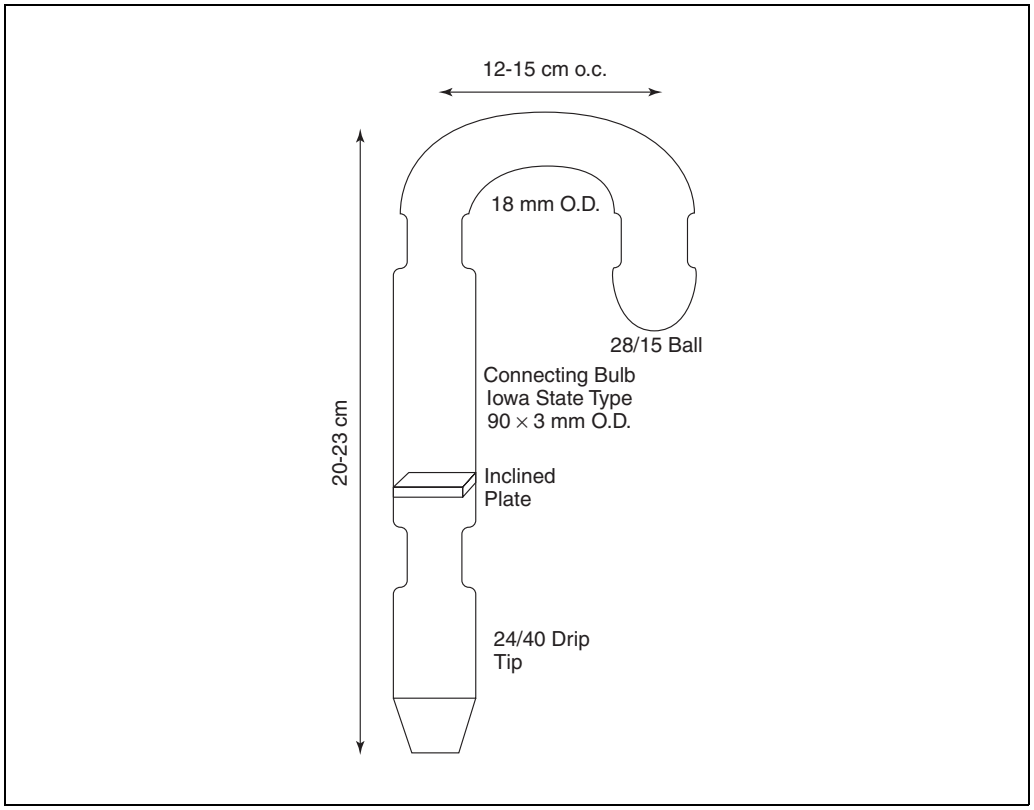

Figure G1.5.4 Depiction of connecting tube adapter for direct distillation. Reprinted from Scott and Veldhuis (1966) with permission from AOAC International.

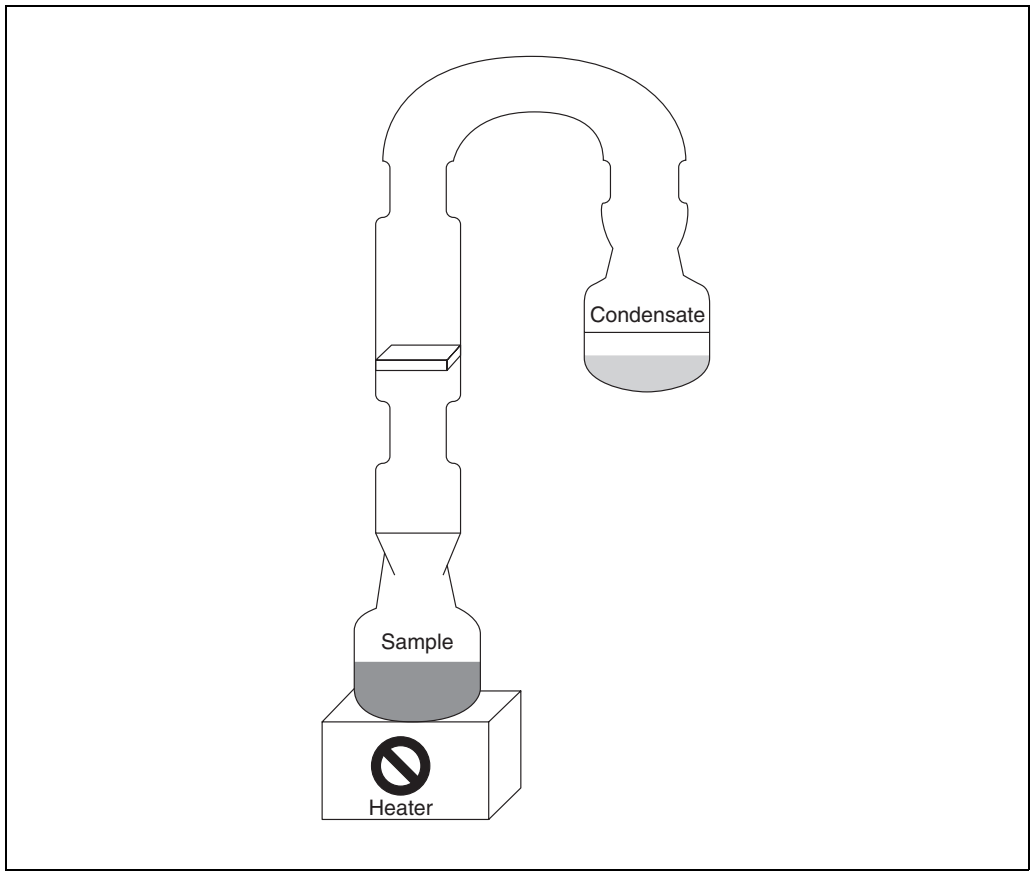

Figure G1.5.5 Schematic diagram for distillation procedure.

QUANTIFICATION OF PRESS CAKE OIL

ALTERNATE PROTOCOL 1

The recovery of cold-pressed oil is not 100% effective. Most oil recovery units capture between 50% and 70%, depending on the type of unit. After the juice is extracted, the wet peel is sent to the feed mill. The peel is treated with 0.3% lime and sent through a shredder. The peel is then pressed, generating press cake and press liquor. The amount of oil in the press cake is critical to volatile organic compounds (VOC) emission levels. This quantitative method is based on the Scott oil analysis described in Basic Protocol 7.

Additional Materials *(also see Basic Protocol 7)*

Press cake sample

1. Perform analysis as described (see Basic Protocol 7, steps 1 to 7) but use 300 g press cake (PC) with 2700 g distilled water.
2. Calculate oil quantity from the press cake sample using the equation:

   g oil/g PC = (x ml 0.1 N titrant/25 g sample) × (3000 g homogenate/300 g PC) × (0.004 ml oil/ml titrant) × (0.84 g oil/ml oil)

3. Convert to lb. oil/ton dry solids using the equation:

   lb. oil/ton dry solids = (lb. oil/lb. PC) × (2000 lb. PC/ton PC) × (y ton PC/ton dry solids)

   where y ton PC/ton dry solids is the inverse of % solids, measured in step 1 of Basic Protocol 7.

QUANTIFICATION OF OIL IN DRY PEEL AND PELLET

ALTERNATE PROTOCOL 2

The amount of oil released from the press cake during drying is at a constant rate, 0.86 kg oil/1 kg press cake (Gentry, 2001). The volatiles released from the press cake are then vented through the waste heat evaporator during concentration of the molasses. The difference in limonene concentration between the press cake and the dry peel is the maximum amount of VOC emissions. The concentration in the press cake is determined from the Scott oil analysis used in Basic Protocol 7.

Additional Materials *(also see Basic Protocol 7)*

Dry peel or pellets

1. Perform analysis as described (see Basic Protocol 7, steps 1 to 7) but use 100 g dry peel/pellets (DP) with 2900 g distilled water.
2. Calculate the oil from the dry peel sample using the equation:

   g oil/g DP = (x ml 0.1 N titrant/25 g sample) × (3000 g homogenate/100 g DP) × (0.004 ml oil/ml titrant) × (0.84 g oil/ml oil)

3. Convert to lb. oil/ton dry solids using the equation:

   lb. oil/ton dry solids = (lb. oil/lb. DP) × (2000 lb. DP/ton DP) × (y ton DP/ton dry solids)

   where y ton DP/ton dry solids is the inverse of % solids, measured in step 1 of Basic Protocol 7.

ALTERNATE PROTOCOL 3

QUANTIFICATION OF OIL IN PRESS LIQUOR AND MOLASSES

During the concentration of the press liquor to molasses, *d*-limonene is distilled and recovered. The recovery of *d*-limonene is a function of evaporator capacity. Most citrus processors add the molasses back onto the wet peel. The difference between the press liquor and the molasses is the amount of distilled limonene. This is determined by Scott oil analysis.

Additional Materials *(also see Basic Protocol 7)*

0.0247 N $KBr/KBrO_3$ solution (see recipe)
Press liquor (PL) or molasses
Additional reagents and equipment for determination of °Brix (*UNIT H1.4*)

1. Determine the soluble solids (°Brix) with a refractometer (*UNIT H1.4*).
2. Perform analysis as described (see Basic Protocol 7, steps 2 to 7), but use 100 g solids and bring to 3000 g total weight, and reduce concentration of titrant to 0.0247 N.

   The normality of the titrant is lower due to the lower limonene content. It is more of an issue for molasses.

   The appropriate amounts of sample and water are determined from the desired amount of solids and the Brix measurement. For 100 g solids from an 18°Brix press liquor, the homogenate would consist of 100 g solids × (1/0.18) = 556 g press liquor and 2444 g water.

3. Calculate the oil from press liquor using the equation:

   g oil/g PL = (x ml 0.0247 N titrant/25 g sample) × (3000 g homogenate/m g PL) × (0.001 ml oil/ml titrant) × (0.84 g oil/ml oil)

4. Convert to lb. oil/ton dry solids using the equation:

   lb. oil/ton dry solids = (lb. oil/lb. PL) × (2000 lb. PL/ton PL) × (y ton PL/ton dry solids)

   where y ton PL/ton dry solids is the inverse of °Brix, measured in step 1.

SUPPORT PROTOCOL 1

DETERMINATION OF MOISTURE CONTENT

Basic Protocol 7 was developed to estimate the mass balance of *d*-limonene within a citrus processing plant. However, the calculations must be normalized to provide a basis of comparison. This is achieved by determining the moisture content of those samples. Samples are dried at low temperatures under a vacuum. This removes the moisture in the sample and prevents any decomposition of the remaining solid matter.

Materials

Sample
Metal weighing dishes
58°C vacuum oven

1. Record weight of metal dish (w_{dish}).
2. Place 10 g sample into metal dish. Record wet total weight (w_{total}).
3. Place overnight into a 58°C vacuum oven.
4. Weigh sample for dry total weight (w'_{total}).
5. Calculate the percent solids and percent moisture using the following equations:

   $w_{total} - w_{dish} = w_{wet}$

   $w'_{total} - w_{dish} = w_{dry}$

   $(w_{wet} - w_{dry})/w_{wet} \times 100 = \%$ moisture

   $100 - \%$ moisture $= \%$ solids

Table G1.5.1 Protocol Modifications Based on Different Types of Citrus Oils[a]

Sample	Amount (g)	Time (min)	CF (g/ml KOH)
Lemon	10	15	0.0761
Lime	10	15	0.0781
Tangerine	20	30	0.0781
Orange	20	30	0.0781
Grapefruit	20	30	0.0781

[a]Data from Redd et al. (1986). CF, correction factor.

QUANTIFICATION OF TOTAL ALDEHYDES IN CITRUS OILS BY HYDROXYLAMINE TITRATION

BASIC PROTOCOL 8

Aldehyde content is considered an important flavor note included in the standard of identities for citrus oils. The flavor strength of an oil is based on the aldehyde content, where higher is better. The two major aldehydes are acetaldehyde and octanal. The quantification of aldehydes is based on the reaction of citral in the sample with a hydroxylamine solution, followed by titration with KOH in the presence of ethyl orange indicator. It is modified from AOAC Method 955.32 (AOAC, 1990c; Redd et al., 1986). This method was originally developed for lemon oils; however, it is applicable to other citrus oils.

Materials

Citrus oil
0.5 N hydroxylamine solution (see recipe)
0.5 N KOH in 60% (v/v) ethanol

1. Weigh 10 to 20 g sample into a 150-ml beaker. Record exact weight.

   For exact amounts of different citrus oils see Table G1.5.1.

2. Add 35 ml of 0.5 N hydroxylamine solution; swirl occassionally for 15 to 30 min (Table G1.5.1).
3. Titrate with 0.5 N KOH to pH 3.5.
4. Calculate the total amount of aldehydes in lemon oil using the correction factor 0.0761 g citral/1 ml 0.5 N KOH; for other oils, use the correction factor 0.0781 g decanal/1 ml 0.5N KOH.

$$\% \text{ aldelhyes} = (x \text{ ml titrated}) \times \text{CF} \times 100\ \% \times (1/\text{oil weight})$$

QUANTIFICATION OF TOTAL ALDEHYDES IN LEMON OIL BY ACID/BASE TITRATION

ALTERNATE PROTOCOL 4

This procedure measures citral content in lemon oil using acid/base titration. It has been modified from the hydroxylamine method, and is from AOAC Method 955.38 (AOAC, 1990d).

Materials

Lemon oil
10% (w/v) phenylhydrazine in absolute ethanol
Benzene
Methyl yellow indicator: 0.1% (w/v) *p*-dimethylaminoazobenzene in absolute ethanol
0.5 N *p*-toluenesulfonic acid in absolute ethanol

1. Weigh 15 g lemon oil sample into 125-ml flask.
2. Pipet 10 ml of 10% phenylhydrazine solution. Let stand 30 min at room temperature.
3. Add 25 ml benzene.
4. Add 0.2 ml methyl yellow indicator. Titrate with 0.5 N *p*-toluenesulfonic acid to an endpoint of pH 3.5.
5. Titrate a separate 10-ml sample of phenylhydrazine solution with 0.5 N *p*-toluenesulfonic acid to an endpoint of pH 3.5.
6. Calculate the amount of total citral using the equation:

   citral (g) = (*x* ml titrant for sample – *y* ml titrant for phenylhydrazine) × 0.076

   and convert to % citral in the 15-g sample.

ALTERNATE PROTOCOL 5

QUANTIFICATION OF TOTAL ALDEHYDES IN ORANGE AND GRAPEFRUIT OIL USING *N*-HYDROXYBENZENESULFONAMIDE

The *N*-hydroxybenzenesulfonamide (HBS) test is a spectrophotometeric assay for determining total aldehydes in orange and grapefruit oil. The method is based on the reaction between HBS and the aldehydes in the oil. HBS contains a secondary amine that reacts with the carbonyl carbon from the aldehyde. This reaction creates an enamine that is highly conjugate and resonance stable. The addition of KOH and $FeCl_3$ prevents the removal or addition of functional groups from destabilizing the structure. The compound has a maximum absorptivity at 525 nm that is quantifiable according to the Beer-Lambert law ($A = \varepsilon cl$). A standard curve using octanal and 2-hexenal is generated. From the standard curve, the aldehyde concentration is extrapolated. This reaction is specific for aldehydes and is more accurate than the hydroxylamine protocol.

Materials

Orange or grapefruit oil
Isopropanol
1.5% (w/v) hydroxybenzenesulfonamide (HBS) in 99% (v/v) isopropanol
1.0 N KOH
1.0% (w/v) ferric chloride ($FeCl_3$) in 2 N HCl
Octanal
2-Hexenal
Spectrophotometer with 1-cm-pathlength cuvettes

Measure total aldehyde content in sample

1. Dilute 0.2 ml of orange or grapefruit oil in 100 ml isopropanol.
2. In triplicate tubes, add 1.0 ml HBS solution to 10 ml diluted sample.
3. Add 1.0 ml of 1 N KOH. Mix solution and let stand for 10 min at room temperature.
4. Add 1.0 ml of 1.0% $FeCl_3$ solution. Mix and let stand for 10 min.
5. Place sample in 1-cm-pathlength cuvette and measure absorbance at 525 nm in a spectrophotometer.

Measure standards

6. Dilute 0.25 g octanal to 250 ml with isopropanol in a volumetric flask.
7. Dilute 0.25 g of 2-hexenal to 250 ml with isopropanol in a volumetric flask.

8. Mix the two solutions in a 500-ml flask to produce a 1000 ppm aldehyde stock solution.
9. Prepare standards using the following dilution scheme:

   1.0 ml aldehyde stock + 99 ml isopropanol = 10 ppm
   2.0 ml aldehyde stock + 98 ml isopropanol = 20 ppm
   3.0 ml aldehyde stock + 97 ml isopropanol = 30 ppm
   5.0 ml aldehyde stock + 95 ml isopropanol = 50 ppm.

10. Repeat steps 1 to 5 using standards.

Calculate aldehyde content of sample

11. Average triplicate values of the standards and generate a standard curve by plotting A_{525} versus concentration.
12. Average triplicate values of the sample and calculate aldehyde concentration (in ppm) by linear regression from the standard curve:

    [aldehyde] = (A_{525} – intercept)/slope

13. Calculate percent aldehyde using the following equations:

    % aldehyde in orange oil = reading from curve × 529 × 100

    % aldehyde in grapefruit oil = reading from curve × 522 × 100

DETERMINATION OF VOLATILE ESTERS

BASIC PROTOCOL 9

Esters contribute to the "fruity" aroma of citrus oils. Esters can be converted to hydroxamic acid with alkaline hydroxyamine. This reaction complexes with ferric chloride, which can be measured colorimetrically.

Materials

2 M hydroxylamine hydrochloride
Citrus oil sample
3 N NaOH
4 N HCl
10% (w/v) ferric chloride ($FeCl_3$) in 0.1 N HCl
Ethyl acetate
Spectrophotometer with 1-cm-pathlength cuvettes

Measure ester content in sample

1. In triplicate tubes, add 2.0 ml of 2 M hydroxylamine hydrochloride to 5 ml citrus oil sample.
2. Add 2.0 ml of 3 N NaOH. Mix solution and let stand for 5 min at room temperature.
3. Add 2.0 ml of 4.0 N HCl.
4. Add 2.0 ml of 10% $FeCl_3$ solution.
5. Place sample in a 1-cm-pathlength cuvette and measure absorbance at 525 nm in a spectrophotometer.

Measure standards

6. Dilute 1.000 g ethyl acetate to 1000 ml with water in a volumetric flask to make a 1000 ppm standard stock solution.

   Flasks must be sealed due to the volatility of ethyl acetate.

7. Prepare a dilution series using the following scheme:

   0.5 ml stock + 99.5 water = 5 ppm
   1.0 ml stock + 99.0 water = 10 ppm
   2.5 ml stock + 97.5 water = 25 ppm
   5.0 ml stock + 95.0 water = 50 ppm
   10.0 ml stock + 90.0 water = 100 ppm
   12.5 ml stock + 87.5 water = 125 ppm
   15.0 ml stock + 85.0 water = 150 ppm
   20.0 ml stock + 80.0 water = 200 ppm.

8. Repeat steps 1 to 5 using standards.

Calculate ester content of sample

9. Average triplicate values of the standards and generate a standard curve by plotting A_{525} versus concentration.

10. Average triplicate values of the sample and calculate the ester concentration (in ppm) by linear regression from the standard curve:

    $[\text{ester}] = (A_{525} - \text{intercept})/\text{slope}$

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Arsenious oxide (As_2O_3) solution

Dissolve 5 g As_2O_3 in 50 ml of 1 N NaOH. Add 50 ml of 1 N H_2SO_4 to adjust pH to ~7.0. Dilute to 1 liter with distilled water. Store up to 7 days at 8°C.

This recipe is from AOAC Method 939.12 (AOAC, 1990a).

Bromphenol blue indicator

Dissolve 0.2 g bromphenol blue in 5 ml of 0.05 N NaOH. Dilute to 100 ml with 60% (v/v) ethanol. Store up to 7 days at 8°C.

Hydroxylamine solution, 0.5 N

Dissolve 34.75 g $H_2NOH{\cdot}HCl$ in 40 ml of hot water and make up 1 liter with 95% ethanol. Determine pH of solution and adjust to pH 3.5. Store up to 7 days at 8°C.

Potassium bromide/bromate ($KBr/KBrO_3$) solution, 0.1 N

Dissolve 2.8 g $KBrO_3$ and 12 g KBr in boiling water and dilute to 1 liter (0.1 N solution). Dilute 125 ml 0.1 N solution with 500 ml water (final 0.0247 N).

Solutions are shelf stable at room temperature. If solutions have not been used for an extended period (6 to 8 weeks), standardize solutions prior to usage.

To standardize the solution, add 40 ml As_2O_3 solution (see recipe) to a 300-ml Erlenmeyer flask. Add 10 ml HCl and 3 drops methyl orange. Titrate with $KBr/KBrO_3$ solution.

Normality $KBr/KBrO_3$ = (ml As_2O_3 × normality As_2O_3)/(ml $KBr/KBrO_3$)

This recipe is from AOAC Method 947.13 (AOAC, 1990b).

Table G1.5.2 Market Value and Limonene Content of Various Citrus Oils[a]

Citrus oil	*d*-Limonene (%)	Market value (U.S. $/gallon)
d-Limonene	100	0.45
Cold-pressed Valencia	>95	0.77
Cold-pressed early-mids	>95	0.65
5× orange oil (Valencia)	90	16.00
10× orange oil	80-85	NA
25× orange oil	60-65	NA
36× orange oil	1-2	NA
Tangerine	95	14.00
Mandarin (Italian)	95	35.00
Grapefruit	93-95	14.00
Lemon (Californian)	75-80	9.50
Lemon (Italian)	75-80	22.75
Lime (Mexican)	50-55	25.50

[a]Adapted from Braddock (1999) with permission from John Wiley & Sons, Inc.

COMMENTARY

Background Information

The diversification and reactivity of oil components can decrease functionality and value. Table G1.5.2 lists the market value for most citrus oils. Prices range from $0.45 per gallon for *d*-limonene to $35.00 per gallon for Sicilian Mandarin. Figure G1.5.6 presents chemical structures of compounds that are important to the flavor of citrus oils.

Orange oils (early-mid/Valencia)

The desired orange flavor is the result of volatile compounds in specific proportions (Shaw, 1991). There are six major contributors to orange flavor: acetaldehyde, citral, ethyl butanoate, *d*-limonene, nonanal, octanal, and α-pinene with two major types of essence oils, early-mid and Valencia (Shaw, 1991). Early-mid oranges include Hamlin and Pineapple.

More than 200 different compounds have been identified in cold-pressed Valencia oil (Maarse and Visscher, 1989). Table G1.5.3 is a list of over 100 compounds identified in cold-pressed Valencia oil. *d*-Limonene is the predominant terpene present in citrus oil. It has a weak "citrus-like" aroma; however, at high levels (190 ppm) it is detrimental to orange flavor. It is believed to be a carrier of minor oil-soluble flavor compounds (Shaw, 1991). The second most abundant terpene is myrcene. Myrcene has long been known to impart a negative characteristic. It has been shown to give a pungency or bitterness at high levels. α-Pinene is another monoterpene that contributes to orange flavor. However, the most notable difference between orange oils is the percentage of valencene. Valencene is a sesquiterpene hydrocarbon. In cold-pressed Valencia oil, it is 10 to 20 times higher than other orange oils (Coleman et al., 1969).

Aldehyde content in orange oil is vital. Acetaldehyde is considered the major "freshness" compound. It has a low threshold concentration (3 ppm) and is often added back to reconstituted frozen concentrate. Citral is another important aldehyde. Citral is an isomeric mixture of neral and geranial. It is typically used at 40 ppm in synthetic orange flavorings. Other aldehydes along with esters and alcohols play a significant role in orange flavor. Ethyl butanoate has a strong, pleasant, fruity aroma with a detection level of 0.4 ppm. Linalool and α-terperineol also contribute to orange flavor, but like other compounds become objectionable at high levels.

The term folded oils refers to concentrated oils. This typically involves a distillation process; however, alcohol washing can also be used. Alcohol washing is based on the insolubility of *d*-limonene in 60% to 70% ethanol. These processes predominately remove terpene compounds, although aldehydes (octanal) are also reduced. Oils that are more than 20-fold concentrated are called "terpeneless oils" and are more stable. Distillation is predominately used by flavor houses. Flavor houses purchase cold-pressed oil, which is concentrated and fractionated. These fractionated portions are sold for flavorings or flavor precursors.

Table G1.5.3 Volatile Compounds Associated with Cold-Pressed Valencia Oil[a]

Acids	Alcohols	Aldehydes	Esters	Ketones	Oxides	Terpenes
Acetic	Amyl alcohol	Acetaldehyde	Bornyl acetate	Acetone	*cis*-Limonene oxide	δ-Cadinene
Capric	Borneol	Citral geranial	Citronellyl acetate	Carvone	*trans*-Limonene oxide	Camphene
Caprylic	*cis*-Carveol	Citral neral	Decyl acetate	α-Ionone		Δ-3-Carene
Formic	*trans*-Carveol	Citronellal	Ethyl acetate	Methyl heptenone		Caryophyllene
	Citronellol	*n*-Decanal	Ethyl butanoate	6-Methyl-5-hepten-2-one		α-β-Copaene
	n-Decanol	*n*-Dodecanal	Ethyl 3-hydroxy hexanoate	Nootkatone		α-β-Cubebene
	Dodecanol	Dodecene-2-al-1	Ethyl isovalerate	Piperitenone		*p*-Cymene
	Elemol	Formaldehyde	Ethyl 2-methyl butanoate			β-Elemene
	Ethyl alcohol	Furfural	Ethyl proprionate			Farnesene
	Geraniol	*n*-Heptanal	Geranyl acetate			α-β-Humulene
	Heptanol	*n*-Hexenal	Geranyl butyrate			*d*-Limonene
	Hexanol-1	trans-Hexen-2-al-1	Geranyl formate			2,4-*p*-Methediene
	Isopulegol	*n*-Nonanal	Linalyl acetate			Myrcene
	Linalool	*n*-Octanal	Linalyl propionate			α-Phellandrene
	cis-trans-2,8-*p*-Menthadiene-1-ol	(*E*)-2-Pentenal	1,8-p-Methadiene -9-yl acetate			α-β-Pinene
	1,8-*p*-Methadiene-1,2-diol	*Perillydehyde*	Methyl butanoate			Sabinene
	8-*p*-Methene-1,2-diol	α-Sinsensal	Neryl acetate			α-Terpinene
	1-*p*-Methene-9-ol	β-Sinsensal	Nonyl acetate			β-Terpinene
	Methyl alcohol	*n*-Undecanal	n-Octyl acetate			α-Terpinolene
	Methyl heptanol		Perillyl acetate			α-Thujene
	Nerol		Terpinyl acetate			Valencene
	n-Nonanol					
	n-Octanol					
	α-Terpineol					
	Terpinen-4-ol					
	Undecanol					

[a]Data from Kesterson et al. (1971) and Shaw (1991).

Mandarin/tangerine oil

Mandarin oranges encompass a wide variety of loose-skin citrus cultivars. In the U.S., the term tangerine is interchanged with mandarin. Tangerines are a deeper colored orange. There is a distinct aroma difference between tangerine and Sicilian mandarin oil. However, the value of mandarin oil versus tangerine oil is significant. Mandarin oil (Sicilian) is the most expensive. Similar to other citrus oils, large levels of terpenes are present. *d*-Limonene values range from 65% to 94% in mandarin oil. However, at lower levels of limonene, there is an increase in γ-terpinene.

The two major components responsible for mandarin flavor are methyl-*N*-methylanthranilate and thymol. These have been based on threshold levels for methyl-*N*-methylanthra-

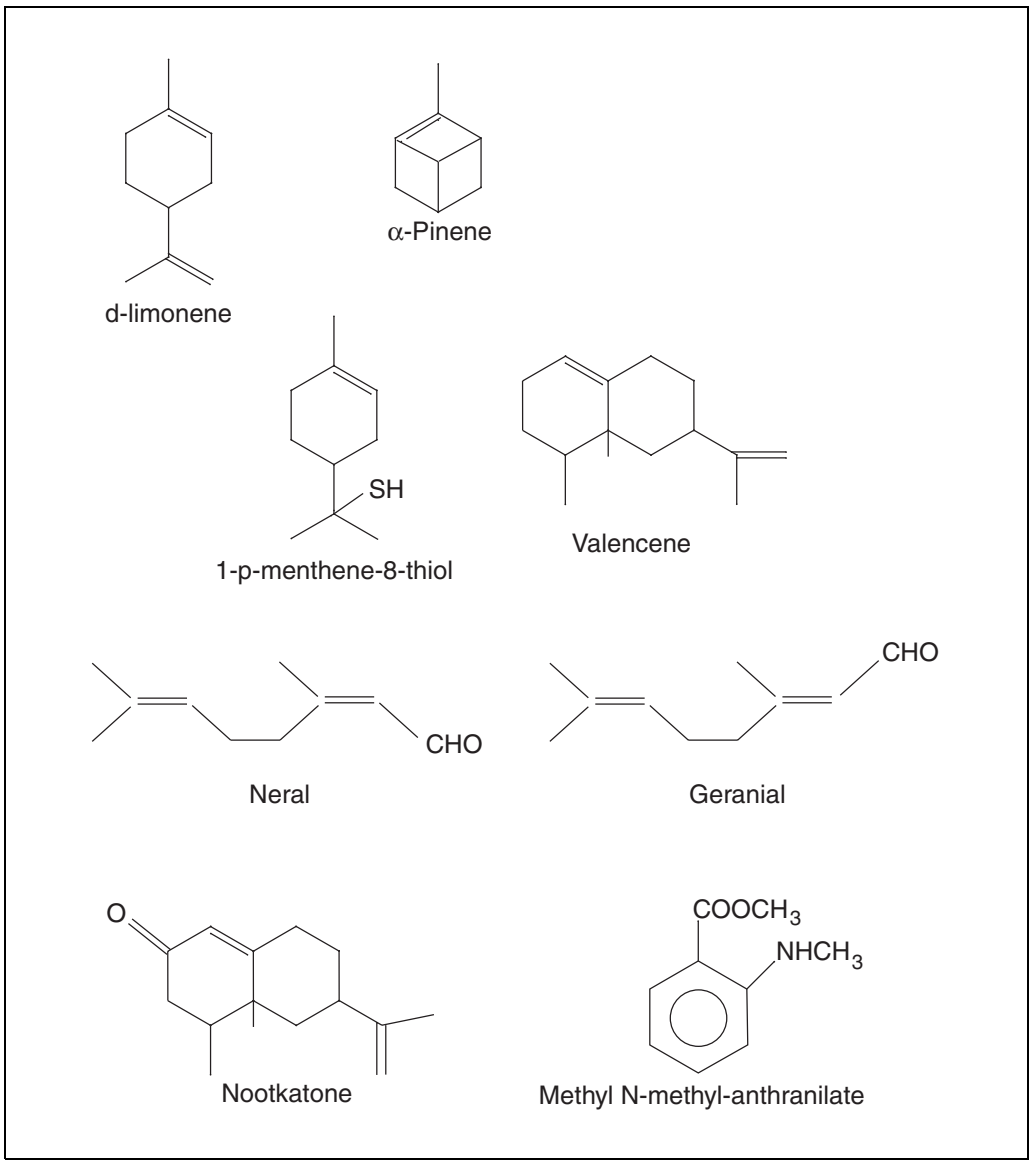

Figure G1.5.6 Chemical structures of compounds important to citrus flavor.

nilate. Methyl-*N*-methylanthranilate is five times higher in Sicilian mandarin oil than Dancy tangerine. In addition, β-pinene and γ-terpinene must be increased in tangerine oil to produce a "mandarin" flavor. Table G1.5.4 compares the major flavor components for mandarin and tangerine oil. Decanal, octanal, α-sinensal, and acetaldehyde are also important to mandarin flavor.

Grapefruit oil

Grapefruit, typically, has harsher flavor notes. Similar to other citrus oils, grapefruit oil is predominately composed of terpenes. There have been 206 volatile compounds identified in grapefruit juice. However, the flavor impact compounds are more dominant. Nootkatone and 1-*p*-methene-8-thiol are the major components for grapefruit oil. Nootkatone is a sesquiterpene ketone that is pungent and aromatic. Sensory panels described samples with high levels of nootkatone as having a lingering bitterness with a metallic taste (Berry et al., 1967). This occurs at 9 ppm. Nootkatone has a flavor threshold in water of 1 ppm and 5 to 6 ppm in grapefruit juice. It has been suggested nootkatone be used as a quality index for grapefruit oil. However, 1-*p*-methene-8-thiol has been described as the "aroma of fresh grapefruit juice" (Demole et al., 1982). There are also significant aldehyde levels, 1.8% (Shaw, 1991).

Lemon oil

The aroma of lemon oil, like other citrus oils, is dependent upon the proportions of specific compounds. Lemon oil has a higher aldehyde

Table G1.5.4 Comparison of the Major Flavor Component for Mandarin and Tangerine Oranges[a]

Component	Tangerine oil	Mandarin oil
Methyl-*N*-methylanthranilate	0.07%	0.65%
β-Pinene	0.17%	1.80%
γ-Terpinene	1.74%	14.00%
Thymol	0.02%	0.18%

[a]Reprinted from Wilson and Shaw (1981) with permission from the American Chemical Society.

Table G1.5.5 Volatile Compounds Important to Lemon Flavor[a]

Alcohols	Aldehydes	Esters	Ethyl ethers	Terpenes
α-Bisabolol	Geranial	Geranyl acetate	Carvyl	Bergamotene
Geraniol	Neral	Methyl epijasmonate	8-*p*-Cymenyl	Caryophyllene
		Neryl acetate	Fenchyl	β-Pinene
			Myrcenyl	γ-Terpinene
			α-Terpinyl	

[a]From Shaw (1991) by courtesy of Marcel Dekker, Inc.

Table G1.5.6 Chemical Compounds Identified in Lime Oils[a]

Alcohols	Aldehydes	Esters	Ketones	Oxides	Terpenes
Borneol	Acetaldehyde	Ethyl acetate	Acetone	1,4-Cineole	α-Bergamotene
p-Cymene-8-ol	*n*-Decanal	Geranyl acetate	Piperitenone	1,8-Cineole	Camphene
Decanol	Geranial	Neryl acetate		*cis*-Linalool oxide	β-Caryophellene
Ethyl alcohol	Hexenal			*trans*-Linalool oxide	*p*-Cymene
Geraniol	2-Hexanal				Dipentene
cis-3-Hexen-1-ol	Neral				α-Elemene
Isoamyl alcohol	*n*-Nonanal				α-Humulene
Isopropyl alcohol	*n*-Octanal				β-Humulene
Lauryl alcohol	Perillaldehyde				*d*-Limonene
Linalool					Myrcene
cis-*p*-2-Menthen-1-ol					α-Pinene
Methyl alcohol					β-Pinene
2-Methyl-2-butanol					γ-Terpinene
2-Methyl-3-buten-2-ol					Terpinolene
3-Methyl-2-buten-1-ol					
Nerol					
Nonanol					
Octanol					
Terpinene-4-ol					
α-Terpineol					
β-Terpineol					

[a]From Azzouz and Reineccius (1976) with permission from the Institute of Food Technologists.

(2.0% to 13.2.%) content than orange and grapefruit oil (Shaw, 1979). High quality oils will have an aldehyde content between 4% and 5%. Lemon oil also has a lower *d*-limonene level. However, the terpene content is 1% higher than other oils with a wider variety of terpene compounds. In particular, β-pinene and γ-terpinene are generally higher in lemon oil. The predominant esters present are neryl and geranyl. In addition, there is a 5% thymol concentration (Moshonas et al., 1972). Table G1.5.5 lists volatile compounds important to lemon flavor.

Lime oil

Lime oil is similar to lemon oil with a few exceptions. Citral content is higher and octanal is the main straight-chained aldehyde. Neryl and geranyl acetate is also higher. The processing of lime oil, cold-pressed versus distilled, does contribute to noteable differences. During distillation, the oil is in contact with acidic juice for a longer time. Distilled lime has reduced levels of citral, β-pinene, and γ-terpinene and increased amounts of *p*-cymene, terpinen-4-ol, and α-terpineol (Slater and Watkins, 1964). In addition, α-thujene, neral, geranial, decanal, geranyl acetate, neryl acetate, and α and β-elemene are absent in distilled lime oil. However, there is very little varietal difference in cold-pressed lime oils, West-Indian and Mexican (Azzouz and Reineccius, 1976). Table G1.5.6 lists the 54 compounds identified in lime oils.

Total available oil

The reactivity of hydrocarbons in citrus oils has brought considerable attention to citrus processing. Processors are only allowed to emit certain levels of volatile organic compounds (VOC). For this reason, a processor must account for the total available oil. With these procedures, 90% of VOC levels can be accounted for (Gentry, 1999).

Critical Parameters

Qualitative analysis

Depending on the choice of column, flow rates and temperature program are important parameters for the qualitative analysis of citrus oils. It is also important to have the same temperature program for quantifying compounds. Replication of injections for generating a standard curve is vital. Injections should be done on the same day by the same technician. A standard curve with an R^2 value >0.9 is sufficient.

The temperature for measuring physical parameters, specific gravity, refractive index, and optical rotation is critical for obtaining accurate results.

Quantitative analysis

The solutions for measuring total available oil are stable and can be prepared in advance. The weight of the sample is important, especially for the dryer samples, press cake, and pellets. These samples absorb a lot of water. Furthermore, the concentration of the potassium bromide solution may need to be decreased if a sample has an extremely low level of limonene (i.e., press liquor or pellets).

For the colorimeteric assays, stock solutions must be fresh (1 to 3 days) and should be refrigerated. HBS solution is only good for 5 days. The standards should be run each time a series of samples is run.

Table G1.5.7 Physical and Chemical Properties of Citrus Oils[a,b]

Cultivar	SG	η	α	Aldehyde (%)	Ester (%)
Cold-pressed orange oil	0.842-0.846	1.472-1.474	+94-99	1.2-2.5	0.48
Orange essence oil	0.840-0.844	1.471-1.474	+94-99	1.0-2.5	2.12
Mandarin	0.847-0.853	1.473-1.477	+68-78	0.4-1.8	NA
Tangerine	0.844-0.854	1.473-1.476	+88-96	0.8-1.9	1.44
Grapefruit	0.848-0.856	1.475-1.478	+91-96	1.3	3.59
Cold-pressed lime (Mexican)	0.872-0.881	1.482-1.486	+35-41	4.5-8.5	6.98
Lime essence oil	0.855-0.863	1.474-1.477	+34-47	0.5-2.5	NA
Lemon (Italian)	0.849-0.855	1.473-1.476	+57-65.6	3.0-5.5	NA
Lemon (Californian)	0.849-0.855	1.473-1.476	+57-65.6	2.2-3.8	NA
Lemon essence oil	0.842-0.856	1.470-1.475	+55-75	1.0-3.5	NA

[a]Defined by the Food Chemicals Codex (NRC,1981).

[b]Optical activity (α); refractive index (η); specific gravity (SG), and aldehyde content from NRC (1981). Ester content from Kimball (1999).

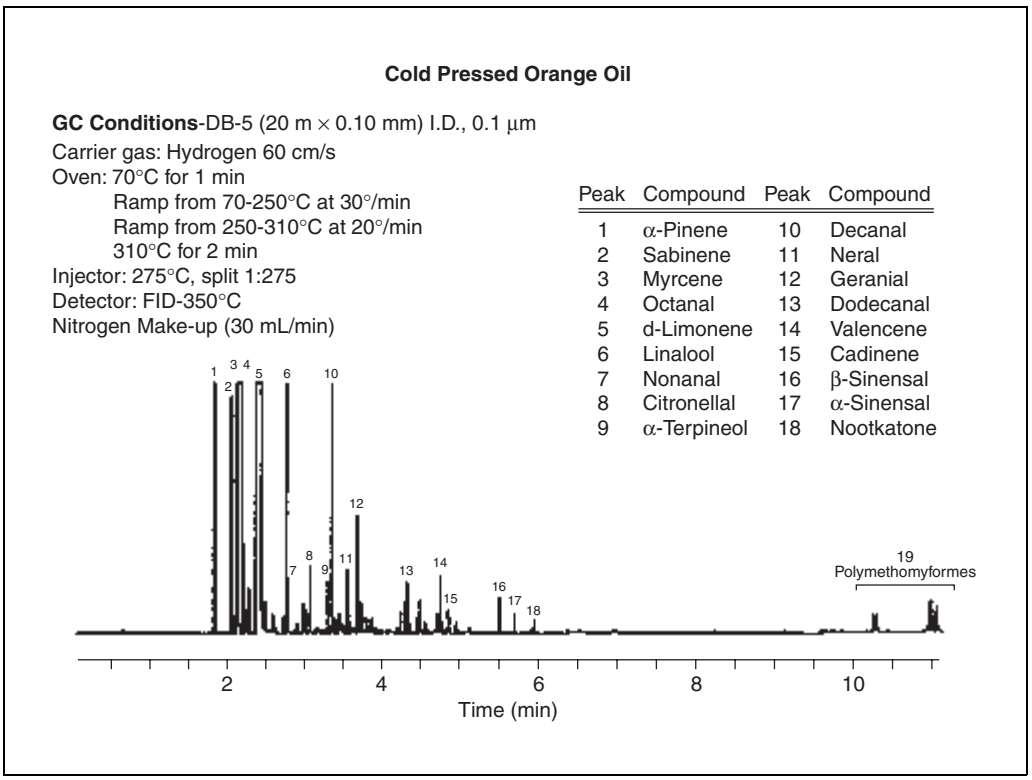

Figure G1.5.7 Typical gas chromatogram for cold-pressed orange oil. Reproduced with permission from Agilent Technologies, Inc. (see Internet Resources).

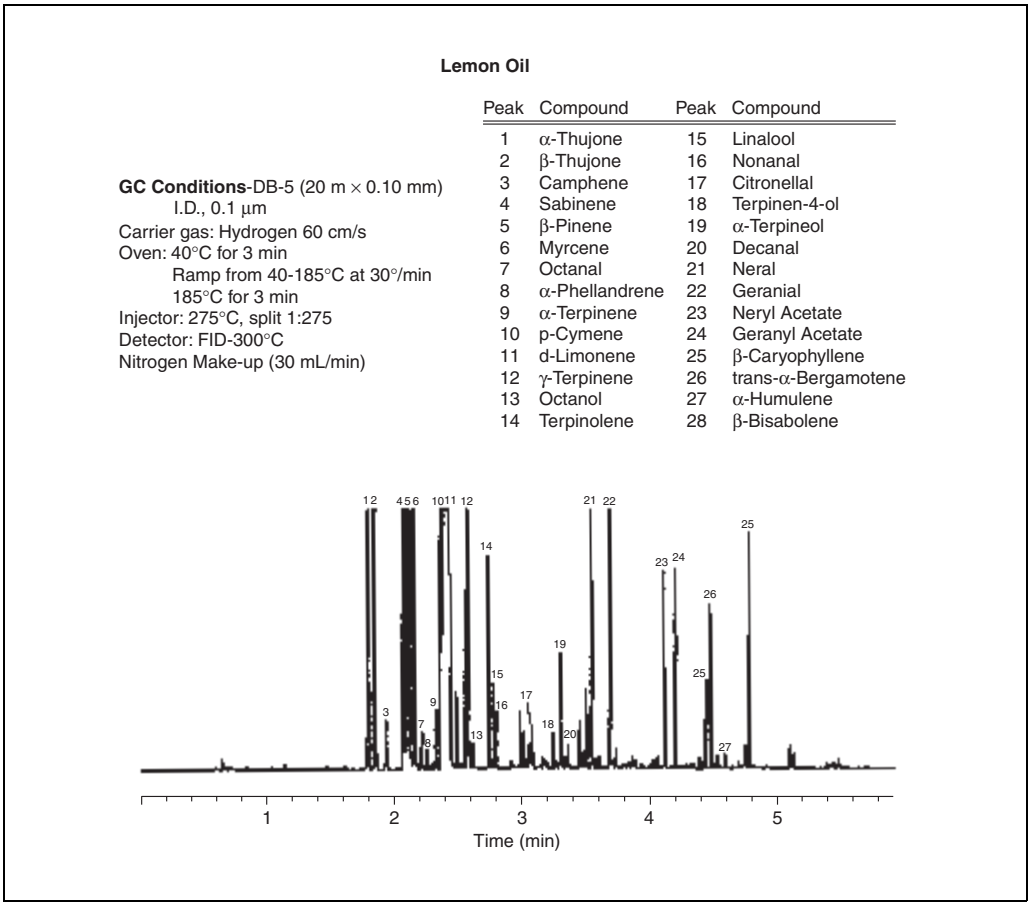

Figure G1.5.8 Chromatogram for lemon oil. Reproduced with permission from Agilent Technologies, Inc. (see Internet Resources).

The volatility of ethyl acetate requires minimal exposure when preparing standards. The same is true for limonene. Limonene should be stored in a metal canister, and the headspace should be flushed with nitrogen after usage.

Troubleshooting

Gas chromatography can be extremely machine-dependent when determining optimal settings. The key is to have reproducibility. Depending on the column, temperature programs may need to be adjusted for better peak resolution. The septa can be a problem. It should be changed regularly if used a lot (~30 injections), otherwise peaks shift retention times. In addition, artifacts can be a problem, especially if the oil has high boiling compounds. Holding the column at a higher temperature for a longer period can eliminate this. If the column continues to show artifacts, setting the column to a high temperature overnight should eliminate the problem. However, this will decrease the life-span of the column. Be sure not to exceed the manufacturer's maximum temperature.

The quantification protocols must be conducted carefully. The concentrations are extremely low. Volumetric flasks and pipets (class A) will provide the most accurate results for preparing standards. Light and oxygen exposure should also be minimized. Oils should be refrigerated in either amber or metal bottles. Plastic should never be used.

Anticipated Results

Table G1.5.7 lists the physical and chemical properties (specific gravity, SG; refractive index, η; optical activity, α) of citrus oils defined by the Food Chemicals Codex (NRC, 1981).

Figure G1.5.7 is a typical chromatogram for cold-pressed orange oil. The peaks are well

Distilled Lime Oil

GC Conditions-DB-5 (60 m × 0.25 mm) I.D., 0.25 µm
Carrier gas: Helium 25 cm/s
Oven: 75°C for 8 min
Ramp from 75-200°C at 4°/min
200°C for 5 min
Injector: 270°C, split 1:150
Detector: FID-270°C
Nitrogen Make-up (30 mL/min)

Peak	Compound	Peak	Compound
1	α-Pinene	16	Bomeol
2	Camphene	17	Terpinen-4-ol
3	β-Pinene	18	α-Terpineol
4	Myrcene	19	γ-Terpineol
5	α-Phellandrene	20	Decanal
6	1,4-Cineol	21	Neral
7	α-Terpinene	22	Geranial
8	p-Cymene	23	Neryl Acetate
9	d-Limonene	24	Geranyl Acetate
10	γ-Terpinene	25	Dodecanal
11	Terpinolene	26	β-Caryophyllene
12	Linalool	27	trans-α-Bergamotene
13	α-Fenchyl Alcohol	28	trans-α-Famesene
14	Terpinen-1-ol	29	β-Bisabolene
15	β-Terpineol		

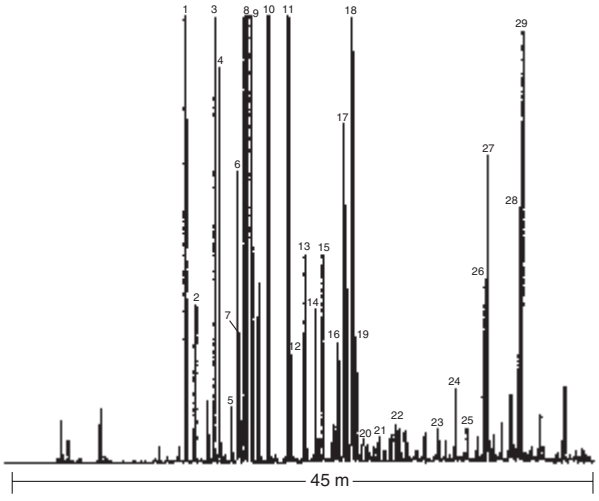

Figure G1.5.9 Gas chromatogram for distilled lime oil. Reproduced with permission from Agilent Technologies, Inc. (see Internet Resources).

Table G1.5.8 Identification of Peaks for Cold-Pressed Versus Distilled Lime Oil[a]

Distilled lime oil		Cold-pressed lime oil	
Peak	Compound	Peak	Compound
1	*tert*-Amyl alcohol	1	*tert*-Amyl alcohol
2	2-Methyl-3-buten-2-ol	2	2-Methyl-3-buten-2-ol
3	*cis*-3-Hexen-1-ol	3	*n*-Nonane
4	*n*-Nonane	4	α-Thujene
5	α-Pinene	5	α-Pinene
6	Camphene	6	Camphene
7	β-Pinene	7	β-Pinene
8	Myrcene	8	Myrcene
9	α-Phellandrene	9	D-Limonene
10	1,4-Cineole	10	1,8-Cineole
11	*p*-Cymene	11	γ-Terpinene
12	D-Limonene	12	Terpinolene
13	1,8-Cineole	13	Octanol
14	γ-Terpinene	14	Linalool
15	Terpinolene	15	Terpinene-4-ol
16	Octanol	16	Decanal
17	Linalool	17	Neral
18	α-Fenchyl alcohol	18	Geranial
19	*cis*-β-Terpineol	19	Decanol
20	*trans*-β-Terpineol	20	Nerol
21	Terpinene-4-ol	21	Geraniol
22	α-Terpineol	22	α-Elemene
23	Neral	23	Thymol
24	Geranial	24	Neryl acetate
25	Decanol	25	Geranyl acetate
26	Nerol	26	β-Elemene
27	Geraniol	27	β-Caryophellene
28	α-Elemene	28	α-Bergamotene
29	Thymol	29	α,β-Humulene
30	Neryl acetate	30	Guaiene
31	Geranyl acetate	31	β-Bisabolene
32	β-Elemene		
33	β-Caryophellene		
34	α-Bergamotene		
35	α,β-Humulene		
36	Guaiene		
37	β-Bisabolene		

[a]From Azzouz and Reineccius (1976) with permission from the Institute of Food Technologists.

defined. The GC conditions are modified for this particular sample.

Hunter and Brogden (1965) generated chromatograms that provide a visual representation of the various citrus oils. The GC conditions for the orange oils are similar, giving the same retention times but different peak ratios for the various hydrocarbons. Comparisons of cold-press Valencia oil from Florida versus California shows a similar qualitative analysis; however, the valencene peak is larger in the California oil. This is not uncommon. Oil samples can show great diversity depending on the degree of oxidation, processing, and storage conditions. Fruit maturity and cultivar will also effect results. Chromatograms for early-mid orange oil also shows different response values compared to Valencia oil. Early-mid oils have

Table G1.5.9 Peel-Oil Content of Various Florida Citrus Cultivars[a]

Cultivar	Average pounds oil/ton fruit	Standard deviation (σ)
Bears lemon	15.1	2.4
Grapefruit	6.2	0.5
Hamlin	7.8	1.5
Persian lime	8.1	1.2
Pineapple	9.7	1.0
Tangerine	15.5	1.8
Valencia	13.5	1.8

[a]From Kesterson and Braddock (1975) with permission from the Institute of Food Technologists.

Table G1.5.10 Aldehyde Composition for Various Citrus Oils[a]

Oil	C_6	C_8	C_{10}	C_{12}	C_{14}	Neral	Geranial
Essence	0.5	13.8	26.7	5.5	1	6	4.2
Grapefruit	Trace	29.9	17.8	12.7	6.9	2.1	7.1
Hamlin	1.2	29.2	22.7	15.1	6.1	1.1	9.3
Pineapple	0.5	28.2	18	9.9	6.5	6.9	10.1
Tangerine	Trace	23.1	24	13.5	4.8	5	5.7
Valencia	1	27.3	30.7	9.4	5.3	4.1	4.4

[a]From Braddock and Kesterson (1976) with permission from the Institute of Food Technologists.

higher amounts of less important hydrocarbons (myrcene, α and β-copene, α and β-ylangene, β-elemene, and caryophyllene). Early-mid oils also have lower valencene peaks. However, for grapefruit oil, the two most noticeable differences are the myrcene and caryophyllene peaks. In tangerine oil, γ-terpinene, Δ, α, and β-elemene are significantly different.

Figure G1.5.8 is a chromatogram for lemon oil. The temperature program is modified compared to that of orange oil.

Figure G1.5.9 is a chromatogram for lime oil. This procedure used a longer column with lower temperature settings. The temperature also increases at a much slower rate. There were a larger number of compounds present compared to other oils. Table G1.5.8 shows peaks for lime oil samples processed under different conditions. The distilled oil has higher levels of alcohol, whereas, the cold-pressed oil has higher amounts of esters and aldehydes.

Table G1.5.9 is a summary of the total available oil for various citrus cultivars. Table G1.5.10 is the aldehyde composition for orange and grapefruit oils.

Time Considerations

Most GC temperature programs will take between 30 and 45 min. The qualitative data is simple once a library of high quality oils is collected. The quantitative data will depend on how many compounds are measured. Each standard curve will take a day to collect reliable data (minimum 12 runs).

The total available oil procedures take ~40 min. It's best to prepare samples before distillation. Samples can be titrated while the next sample is distilled. The other titration procedures should not take >20 min, provided reagents are prepared. The colorimetric assays will take ~3 hr given preparation of reagents and generation of standard curve.

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Internet Resources

http://www.chem.agilent.com/scripts/chromatograms.asp

Agilent Technologies' on-line Chromatogram Library.

Contributed by Trevor Gentry
Cornell University
Ithaca, New York

Solid-Phase Microextraction for Flavor Analysis

A compound must volatilize from a food to reach receptors in the nasal epithelium and potentially produce an aroma perception. Methods that selectively extract volatile compounds from food are advantageous for flavor analysis. Furthermore, because aroma compounds are present at very low concentrations (often below parts-per-billion levels), methods that concentrate these volatiles are necessary. Solid-phase microextraction (SPME) is an extraction method based on absorption and adsorption of compounds to polymers coated on a silica fiber, effectively concentrating analytes found in a gas or liquid phase. The absorbed compounds can subsequently be separated using gas chromatography. SPME is a nondestructive, reproducible method. A variety of fiber-coating materials are used for selective extraction of analytes. For quantification, SPME can be standardized to account for fiber selectivity. SPME can be used in conjunction with mouth simulators (*UNIT G1.7*), gas chromatography/mass spectrometry (GC/MS), gas chromatography/olfactometry (GC/O), and dilution analysis. This unit presents methods for SPME of food headspace (see Basic Protocol) and submersion SPME for liquid samples (see Alternate Protocol). In addition, quantification of headspace extraction (see Support Protocol 1) and dilution analysis (see Support Protocol 2) are presented.

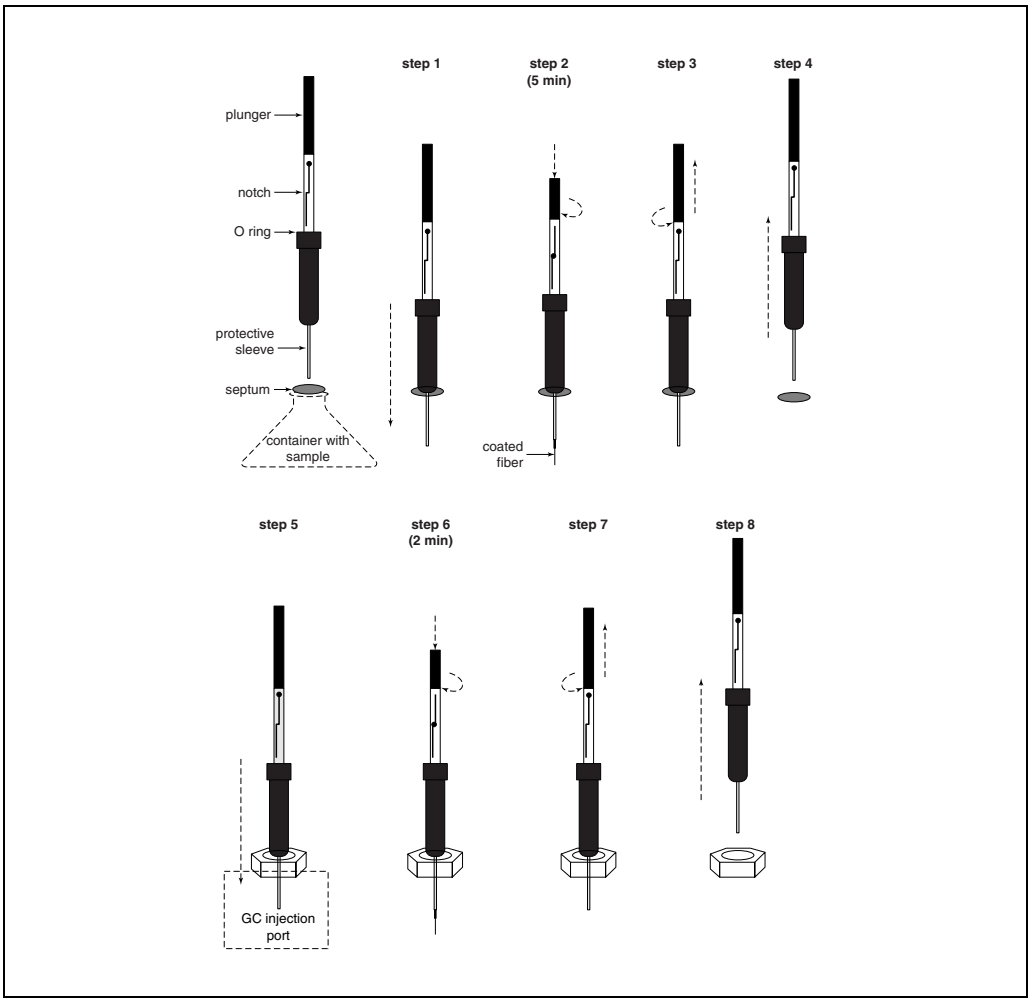

Figure G1.6.1 Illustration of procedures performed in the Basic Protocol for SPME headspace sampling.

Contributed by Kathryn D. Deibler
Current Protocols in Food Analytical Chemistry (2002) G1.6.1-G1.6.12

SPME OF FOOD HEADSPACE FOR GAS CHROMATOGRAPHY

Headspace SPME is a solventless extraction method where a silica fiber coated with adsorbant or absorbant polymer material is exposed to a gas phase to extract analytes. The food of interest is placed in a closed or open container (such as a mouth simulator). After extraction, the fiber is desorbed in a GC injection port for separation and detection of the extracted analytes.

NOTE: Figure G1.6.1 demonstrates the sequence of injection. It is important to follow this sequence carefully to avoid destruction of the fiber. Removing the fiber through a septum without retracting the fiber will strip the coating from the fiber and thus destroy it.

Materials

Food sample for analysis
Headspace vessel (any vessel that can physically support the food sample) with septum (anything that can be pierced by syringe, e.g., Parafilm, aluminum foil, cap septum)
SPME manual assembly (Supelco)
SPME fiber (Supelco), available with different coatings (Table G1.6.1)
GC instrument

1. Place food sample in vessel with septum. Insert SPME through septum.

   See Critical Parameters for discussion of sample size. Closed or open systems may be used; the container need not be sealed.

2. Expose coated SPME fiber to headspace for 5 min, monitoring precisely with a timer.

   Exposure time can be chosen arbitrarily or the time to equilibrium can be used. Time to equilibrium is determined by extracting for incremental times until a stable extracted concentration is achieved. It is imperative that the selected exposure time be used exactly and consistently for any measurements that are to be compared.

3. Retract SPME fiber.

4. Remove the SPME apparatus from the septum.

5. Inject SPME through the septum of a GC for 1.0 min with the purge off and the remaining GC parameters set to the desired analytical conditions.

   Purge flow should be set to ≥30 sec. Longer times are acceptable; 1.0 min is usually used.

6. Expose coated SPME fiber in the injection port. Leave for 2 min.

   Desorption of the analytes takes place in the first minute. The second minute is for cleaning or baking-off purposes. This time of exposure does not need to be carefully monitored and may vary widely, provided that the fiber is exposed in the injection port for at least the length of time that the purge is off.

Table G1.6.1 Selected SPME Polymer Coatings with Recommended Applications

Coating material	Recommended applications
Polydimethylsiloxane (PDMS)	Volatiles, nonpolar semivolatiles
PDMS/divinylbenzene (PDMS/DVB)	Polar volatiles
Carbowax/DVB (CW/DVB)	Polar analytes
PDMS/Carboxen (PDMS/CAR)	Trace level volatiles
Polyacrylate	Polar semivolatiles

7. Retract SPME fiber.
8. Remove SPME apparatus from the injection port.

   Fiber degradation should be monitored after every ten sample runs (see Critical Parameters).

SUBMERSION SPME IN LIQUID SAMPLES

ALTERNATE PROTOCOL

As an alternative to headspace extraction, analytes can be extracted by submersion of an SPME fiber in a liquid sample such as a beverage. While the ratio of analytes in the liquid phase is different from that which would be observed in the corresponding headspace gases, the concentration of most analytes is much higher in the liquid phase. Submersion SPME is most applicable when the basic composition in the food is desired. It is often used as a replacement for solvent extraction.

Additional Materials *(also see Basic Protocol)*

Liquid sample for analysis
Magnetic stirrer and stir bar (or other stirring method)

1. Stir liquid sample at a high and constant rate using a magnetic stirrer and stir bar.

   Viscous samples make mass transfer through the sample slow. Stirring reduces the time for mass transfer.

2. Place an SPME fiber so that the tip is in the liquid.

   For composition analysis, the depth of the fiber does not matter. For comparative analysis, it is important to use a consistent fiber depth.

3. Expose coated SPME fiber to liquid for 30 min, monitoring precisely with a timer.

   Exposure time can be arbitrarily chosen or the time to equilibrium can be used. Time to equilibrium can be determined by extracting for incremental time periods until a stable extracted concentration is achieved. Equilibration time is much greater for submersion extraction than for headspace extraction. It is imperative that the selected exposure time be used exactly and consistently for any measurements that are to be compared.

4. Retract SPME fiber.
5. Remove the SPME apparatus from the liquid.
6. Inject into GC instrument for specific period of time (see Basic Protocol, steps 5 through 8).

QUANTIFICATION OF HEADSPACE EXTRACTION

SUPPORT PROTOCOL 1

Because SPME extracts compounds selectively, the response to each compound must be calibrated for quantification. A specific compound can be quantified by using three GC peak area values from solvent injection, static headspace (gas-tight syringe), and SPME. The solvent injection is used to quantify the GC peak area response of a compound. This is used to quantify the amount of the compound in the headspace. The SPME response is then compared to the quantified static headspace extraction. These three stages are necessary because a known gas-phase concentration of most aroma compounds at low levels is not readily produced. A headspace of unknown concentration is thus produced and quantified with the solvent injection. Calibration must be conducted independently for each fiber and must include each compound to be quantified.

Materials

Volatile compound to be quantified
GC-grade solvent, e.g., ethyl acetate (for solvent injection)
Low-volatizing, nonaqueous solvent: e.g., propylene glycol (for static extraction)
Liquid nitrogen or other cryogenic liquid

GC instrument
100-ml addition funnel without side arm, with standard taper ground-glass joint (24/40) at top and bottom
50-ml round-bottom flask
Exterior thread adapter (24/40) with septum and cap
5-ml gastight syringe (SGE)
500-ml Dewar flask (small enough to fit in GC oven)

Perform solvent injection

1. Prepare a 0.01% (w/v) solution of the volatile compound of interest in a GC-grade solvent.

   This solution can contain more than one compound to be tested. However, if a single compound is used, the purity of the chemical can be measured and taken into account in calculating the actual concentration. Purity of the standard chemicals is important.

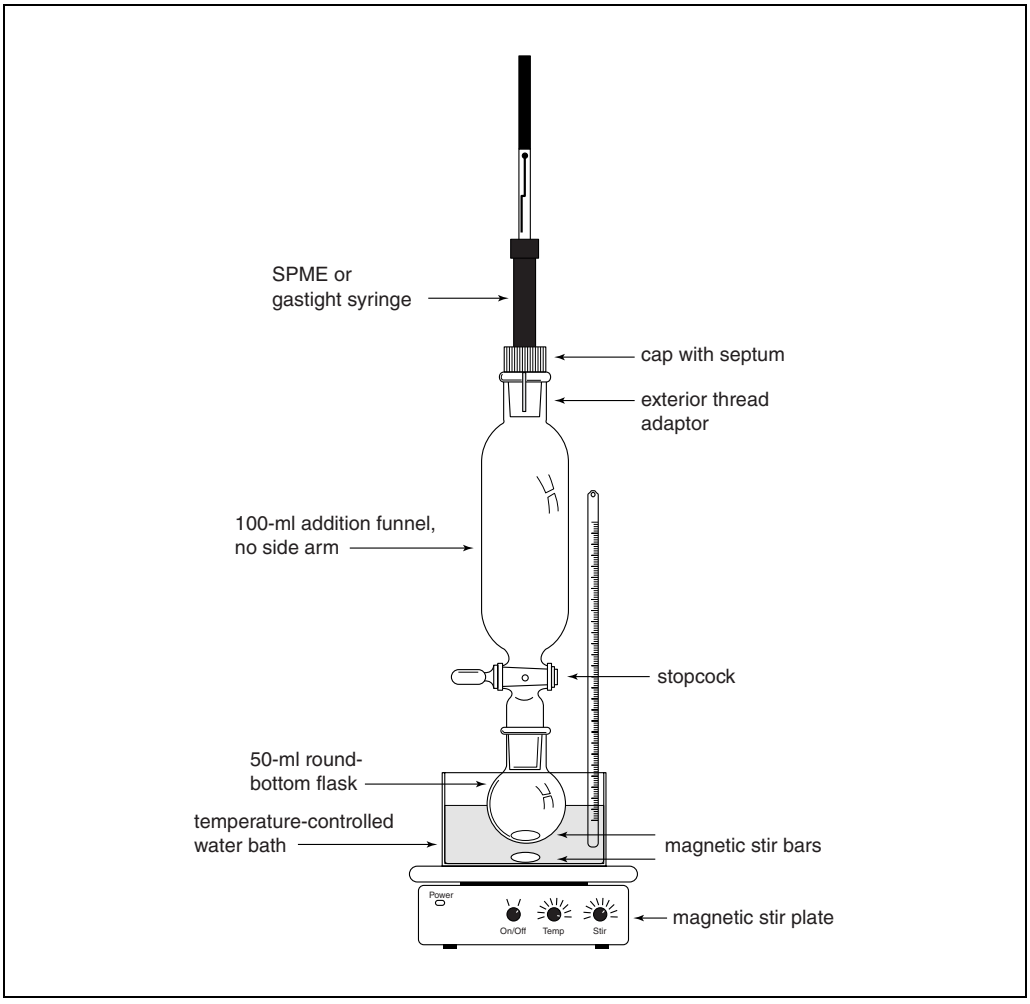

Figure G1.6.2 Apparatus for quantification of headspace extraction in Support Protocol 1.

2. Inject 1 µl of the solution into a GC and analyze according to manufacturer's instructions using the appropriate analytical parameters consistently throughout.
3. Repeat steps 1 and 2 for a total of three measurements and average the peak areas to give the solvent peak area (A_s).

   If the solution contained more than one standard compound, a separate value of A_s is obtained for each compound.

Perform static extraction

4. Set up a 50-ml round-bottom flask, 100-ml addition funnel (with no side arm), threaded adaptor, screw cap with septum, magnetic stir bar, magnetic stir plate, and temperature-controlled water bath as shown in Figure G1.6.2.

   The temperature of the liquid can be controlled with a hot water bath or any other heating or cooling method.

5. Prepare at least 300 ml of ~0.1% (w/v) solution of the volatile compound in a low-volatizing, nonaqueous solvent (e.g., propylene glycol).

   A low-volatility solvent is preferred to reduce solvent interaction in the headspace. More than one solution may be necessary if there is chromatographic overlapping of standard compounds or contaminants.

   The concentration of each volatile must be high enough for gastight syringe extraction, but low enough that the SPME fiber is not saturated and the GC detector limits are not exceeded. An acceptable concentration must be determined empirically by interative application of the two extractions.

6. Transfer 25 ml of solution to the round-bottom flask and stir with the stopcock open for at least 30 min.
7. Close the stopcock to separate the liquid phase in the flask from the gas phase in the addition funnel.
8. Extract 5 ml headspace through the septum with a 5-ml gastight syringe.

   If a cryofocusing unit is installed on the GC column, that should be used instead of the procedures in steps 9 and 11. Cryofocusing freezes the injected compounds at the immersed section, so more sample may be injected over time. Heating of that section results in the transfer or "injection" onto the column.

9. Place approximately the initial loop of the GC column in a Dewar flask containing liquid nitrogen.
10. With purge flow off, inject gas from the syringe into the column at a rate that is equal to or below the flow rate of the column.

    If the column flow rate is 2 ml/min, injection of 5 ml should take at least 2.5 min.

11. Start the GC run using the same parameters used for solvent injection (step 2) and immediately (but carefully) remove the column loop from the cryogenic liquid.

    If the peak area is too small for detection or for reasonable calculations (i.e., <10% of the peak area from sample analysis), the static extraction analysis should be repeated with a more concentrated solution.

12. Repeat steps 6 to 11 for a total of three measurements and average the peak areas to give the vapor peak area (A_v).

    If the solution contained more than one standard compound, a separate value of A_v is obtained for each compound.

Perform SPME

13. Repeat steps 6 and 7.

14. Inject the SPME fiber being calibrated through the septum and measure peak area (see Basic Protocol), but expose the fiber to the headspace for exactly 5 min.

    This is likely to be more than enough time to reach equilibrium, since the liquid phase is separated and the gas phase is relatively large. The time should be measured precisely, e.g., using a digital stopwatch that measures down to a tenth of a second.

    If another GC peak resulting from SPME of the sample or other extractions is larger than the peak observed, the fiber is not saturated. Saturation can be tested (see Critical Parameters). If the fiber is saturated, or if the GC peak is flat at the top or exceeds the detector limits, the static extraction and SPME analysis should be repeated with a less concentrated solution.

15. Repeat steps 13 and 14 for a total of three measurements and average the peak areas to give the SPME peak area (A_{SPME}).

    If the solution contained more than one standard compound, a separate value of A_{SPME} is obtained for each compound.

Analyze data

16. Calculate the calibration constant (k_{SPME}) for each compound calibrated.

    $$k_{SPME} = (C_s V_s / A_s) \times (A_v / V_v) \times (1 / A_{SPME})$$

    where C_s is the concentration of compound in solvent injection (step 1), V_s is the volume of solvent injected (step 2), and V_v is the volume of gas in the gastight syringe (step 8). A_s, A_v, and A_{SPME} are defined in steps 3, 12, and 15, respectively.

    There is a unique k_{SPME} for each compound and fiber phase.

17. Calculate the concentration of standard compound extracted from a headspace by the calibrated SPME (see Basic Protocol) by multiplying the measured peak area by the calibration factor.

SUPPORT PROTOCOL 2

DILUTION ANALYSIS

Gas chromatography/olfactometry (GC/O) based on dilution analysis (e.g., CharmAnalysis or Aroma Extraction Dilution Analysis) gives an indication of what compounds are most potent in the aroma of foods. The application of SPME to GC/O dilution analysis can be achieved by varying the thickness of the fiber phase and the length of exposure, resulting in various absorbant volumes.

Additional Materials *(also see Basic Protocol)*

Gas chromatography/olfactometer (GC/O; DATU, Inc.)
SPME fibers with same coating material but varying thicknesses

1. Perform SPME (see Basic Protocol), but inject into a GC/O.

2. Repeat using a fiber with the same coating material but at approximately one-third the thickness or by exposing only one-third of the original fiber's length. For example, if SPME was originally performed by fully exposing a 1-cm long, 100-µm polydimethylsiloxane (PDMS) fiber, repeat with a 30-µm PDMS fiber or expose only 0.33 cm of fiber.

    The fiber is always fully exposed during desorption in the GC injection port. Fractional exposure can be consistently achieved by either drilling a notch in the injection holder or by restricting the plunger with an O ring. The amount exposed must be measured from the

Table G1.6.2 Comparison of Extraction Methods

Method	Equipment	Sample form	Quantitative	Concentration	Precision
SPME	Holder, fiber	Headspace or liquid	Indirectly	Yes	Very good
Solvent extraction	Solvent, glassware	Solid or liquid	Sample composition	Yes	Excellent
Porous polymer	Gas flow, trap, porous polymer, thermal desorption unit, and cryofocus or solvent	Headspace	Yes	Yes	Good
Purge and trap	Gas flow, trap, porous polymer, thermal desorption unit, and cryofocus or solvent	Liquid	No	Yes	Good
Static headspace	Gastight syringe, cryofocus	Headspace	Yes	No	Fair

fiber, not from the plunger, since the final ~0.5 cm of plunger length is not fiber. It has been observed that the holder must be tightly assembled to ensure consistent exposure amounts.

Commercial nominal thickness is often different from actual thickness. Actual thickness should be requested from the manufacturer or measured under a microscope.

3. Repeat step 1 using a fiber that is one-ninth the original thickness (e.g., 15 µm PDMS) or exposing one-third of a fiber that is one-third the original thickness (e.g., exposing 0.33 cm of 30 µm PDMS).
4. Repeat step 1 exposing one-third of a fiber that is one-ninth the original thickness (e.g., exposing 0.33 cm of 15 µm PDMS).
5. Make potency dilution analysis calculations for GC/O.

See Acree (1997), Acree and Barnard (1994), Acree et al., (1984), and Grosch (1994) for details on GC/O

COMMENTARY

Background Information

SPME

SPME is a sample-preparation technique based on absorption that is useful for extraction and concentration of analytes either by submersion in a liquid phase or exposure to a gaseous phase (Belardi and Pawliszyn, 1989; Arthur et al., 1992). Following exposure of the fiber to the sample, absorbed analytes can be thermally desorbed in a conventional GC injection port. The fiber behaves as a liquid solvent that selectively extracts analytes, with more polar fibers having a greater affinity for polar analytes. Headspace extraction from equilibrium is based on partition coefficients of individual compounds between the food and headspace and between the headspace and the fiber coating (Arthur et al., 1992; Zhang and Pawliszyn, 1993). Zhang and Pawliszyn (1993) give a thorough discussion of the theory of headspace equilibrium extraction. [It has been shown that equilibrium need not be achieved for extraction; instead, a consistent extraction time is sufficient (Ai, 1997).] Removing the solid or liquid sample from the gas phase after analytes have volatilized simplifies the sampling system to only two systems equilibrating: the SPME coating and the gas phase (Roberts et al., 2000). This can be accomplished using an apparatus such as the one shown in Figure G1.6.2.

SPME first found application in evaluating pollutants in water (Belardi and Pawliszyn, 1989). Since then, SPME has been used in an array of fields including the compositional analysis of water, air, essential oils, caffeine,

apple volatiles, pharmaceutical products, insect pheromones, and botanicals (Hawthorne et al., 1992; Malosse et al., 1995; Field et al., 1996; Matich et al., 1996; Guidotti et al., 1999; Elmore et al., 2001; Lee et al., 2001). Several good reviews of SPME have been written (e.g., Eisert and Pawliszyn, 1997; Pawliszyn, 1997).

SPME has been commercially available since 1993 with various adsorbent and absorbant materials and various coating thicknesses. Table G1.6.1 lists some of these coatings and the types of compounds for which they are recommended. Fibers can be made in the laboratory using different polymer coatings (Belardi and Pawliszyn, 1989).

For a compound to contribute to the aroma of a food, the compound must have odor activity and volatilize from the food into the headspace at a concentration above its detection threshold. Since aroma compounds are usually present in a headspace at levels too low to be detected by GC, headspace extraction also requires concentration. SPME headspace extraction lends itself to aroma analysis, since it selectively extracts and concentrates compounds in the headspace. Some other methods used for sample preparation for aroma analysis include purge-and-trap or porous polymer extraction, static headspace extraction, and solvent extraction. A comparison of these methods is summarized in Table G1.6.2.

Solvent extraction

Solvent extraction gives a representation of what is present in the food; however, this is usually quite different from what volatilizes and actually comes in contact with the olfactory epithelium to induce a sensation. Because the exhaustive nature of solvent extraction does not account for volatility of compounds, compounds with low volatility may be determined as making an erroneously large contribution to the aroma if they are present at high concentration. This is often the case with vanillin, which is often present at high concentrations, but has such a low volatility that it may contribute little or not at all to the aroma. A compound with a high volatility, like ethyl butyrate, may be present in the food at a lower concentration, but contribute more to the food's aroma. Potency is also an important factor in determining a compound's contribution to aroma; this is addressed in the literature (Acree, 1997; Acree and Barnard, 1994; Acree et al., 1984; Grosch, 1994). In contrast to solvent extraction, headspace SPME is a method that is selective for compounds that have volatilized from the food. Just as a solvent is selective in its extraction of like compounds, so is the coating in SPME. SPME has replaced solvent extraction in many industries because it is nontoxic and nonhazardous, because disposal is simple, and because it is relatively inexpensive.

Static headspace

Static headspace sampling uses a gastight syringe and a cryogenic liquid such as liquid nitrogen to cryofocus the gas sample on the GC column. The volatilized compounds are extracted in the same ratio in which they are present above the sample, and thus the headspace can be quantified. Gastight syringes must be well monitored for leakage. A precise technique must be replicated to obtain reproducible results. Limited dilution analysis can be achieved using static headspace sampling. Since aroma compounds can be present below parts per billion, the compounds cannot be detected by conventional techniques, with the exception of GC/O. SPME allows for concentration of the compounds in the headspace for analysis. Though the ratio of extraction is distorted by the selectivity of the fiber coating, this can be accounted for by the quantification method in Support Protocol 1. However, these added steps may introduce error. Static headspace is most applicable when the concentration in the headspace is high enough to be detected.

Purge and trap

By having a gas flow over a food sample and then flow through a "trap" containing a porous polymer (e.g., Tenax TA), volatiles are extracted. If the gas flows through a liquid sample, the process is called purge and trap. These headspace methods can be quantitative. Care must be taken to avoid breakthrough of compounds on a saturated polymer. Due to the small diameter of the traps, a low flow rate is usually used to eliminate or reduce back-pressure. Technical difficulties may be encountered in releasing the volatiles from the polymer, especially with thermal desorption units. Washing with solvent masks any compounds that would elute by GC at the same time, which is often the case with diacetyl. Studies have shown that the flow rate is important in effecting the ratio of compounds released in a mouth simulator (Deibler and Acree, 2000). SPME is useful in cases where a higher gas flow rate is desired, such as with a mouth simulator. SPME is quicker and simpler than porous polymer extraction, and usually produces a more reproduc-

ible result. The direct quantitative nature of porous polymer extraction is its primary advantage over SPME. Purge and trap does not quantitatively extract what is in the headspace, but does increase the amount extracted for most compounds due to bubbling through the sample.

Stir-bar sorptive extraction

Stir-bar sorptive extraction (SBSE) is carried out using a commercially available glass stir bar (Twister, from Gerstel GmbH) coated with polydimethylsiloxane (PDMS). A special thermal desorption unit is necessary to introduce the extract into a GC. It can be applied to headspace extraction, but is intended for stirring liquid samples for extraction. The same coatings used for SPME can be used for SBSE, and thus similar selectivity should be observed.

Critical Parameters

Time and temperature

Replication of time and temperature conditions is crucial. Consistent sample conditions must be carefully followed to be able to compare samples. Temperature can be controlled by placing vials in a temperature-controlled water bath or by placing the sample in a jacketed vessel. Time can be carefully monitored using a stopwatch. Consistency in sampling time is imperative; autosamplers do a good job in this respect.

Selection of fiber coating material

Since fiber coating materials have different extraction characteristics resulting in different selectivity, no single fiber is optimal for all situations. Polar coatings tend to be selective for polar compounds, while nonpolar coatings preferentially extract nonpolar compounds. Table G1.6.1 lists some of the fiber coatings that are commercially available and the types of compound for which they are recommended. For optimal fiber selection, the Basic Protocol should be used to evaluate multiple types of coated fibers that represent a range of chemical characteristics or seem appropriate for the class of compounds expected to be present. Comparison of the chromatograms from the tested fibers will allow selection of the fiber or combination of fibers that give the best extraction of the compounds of interest. It is not always important that the peak area be the highest, since this can be accounted for during quantification (see Support Protocol 1); however, greater intensity reduces absolute error.

Conditioning the fiber

Commercial fibers must be conditioned prior to initial use. Each coating requires different conditioning parameters, and the manufacturer's recommendations should always be followed. As an example, conditioning of Supelco's 100-µm polydimethylsiloxane (PDMS) fiber is performed by heating the fiber to 250°C for 1 hour. Conditioning can be carried out in the injection port of a GC or in a special conditioning apparatus.

Cleaning the fiber

Prior to each use, a SPME fiber should be cleaned by exposing it for 2 to 10 min to a temperature that is within the range recommended by the manufacturer for desorption. Since the fiber will have been cleaned during sample desorption, subsequent sampling within a few hours does not require cleaning, provided the fiber is kept retracted and away from high levels of volatiles.

Monitoring fiber degradation

Fiber degradation can result from exposure to heat and solvents, which should be minimized. Leaving a fiber in a hot injection port overnight or for several hours will shorten the life of the fiber. Fiber degradation should be monitored by periodically extracting the headspace of a standard solution containing compounds that are expected in the food sample being analyzed. It is not necessary to use all the compounds of interest. To monitor degradation, a solution containing 0.01% (w/v) of the desired compound(s) in water is placed in a glass container with a septum seal. Any amount of solution and any size container may be used, although larger containers require less time to equilibrate, thus reducing error. The solution amount and container size must be consistent throughout the monitoring process. The container with solution is held in a heated water bath (e.g., 35°C for 1 hr). Any temperature and time can be used as long as they are consistent throughout monitoring. Headspace is analyzed as in the Basic Protocol. The process is repeated after every ten sample runs and chromatograms are compared. If the peak area has been reduced by >5%, the fiber's ability to extract volatiles has been compromised. The fiber should no longer be used for comparative or quantitative purposes, or the fiber degradation effect should be calculated in subsequent sample chromatograms. Once initial monitoring has been performed, degradation can be monitored more or less frequently, depending on the level of deg-

radation caused by the temperature and solvent conditions being used.

Enhancing qualitative extraction

If quantitative extraction is not necessary, some sample conditions may be adjusted to increase volatility of selective compounds. It is important to recognize that the selective nature of these changes in sample conditions affects the *relative* volatility of compounds, and thus distorts the amount of each compound that volatilizes. Thus, quantification of enhanced extractions is not truly representative of the sample's relative headspace.

Commonly referred to as "salting out," adding a salt (e.g., NaCl) to the sample changes the ionic environment and occupies water. Water-soluble compounds concentrate in the available water and equilibrate into the headspace at higher concentrations. Other compounds (insoluble in water) may be unaffected or may decrease in volatility. Changing the pH has a similar effect. Additionally, adjusting the ionic state of compounds will increase or decrease volatility, since ionized compounds do not volatilize.

Heating a sample will increase the total energy, increasing volatility in a manner consistent with the ideal gas law in most cases. However, it was observed that the volatility of terpenes in a beverage is decreased with increasing temperature (Deibler and Acree, 2000). Stirring a sample also adds energy to the system and thus increases volatility.

Monitoring fiber saturation

Saturation of the fiber coating is only a concern for absorptive coatings such as divinylbenzene and carboxen, and only if comparative or quantitative analysis is desired. To verify that the fiber is not saturated, a sample of higher volatile concentration is extracted and analyzed. The resulting chromatogram should show an increased peak area for the compound. If extraction of a more concentrated sample results in the same peak area, the fiber was already saturated at the lower concentration. The amount of increase is not important. Any increase in response is acceptable to show that the fiber is not saturated. When dealing with a beverage or solution, the increased concentration can easily be achieved by simply increasing the volatile ingredients. The headspace concentration can be increased using techniques described above for enhancing qualitative extraction.

Be aware that methods used to increase volatile concentration will affect the volatile ratios; however, that may be compromised when simply looking for saturation. These methods do not increase volatility for all compounds, so if no peak-area increase is observed, it could possibly be because the volatile's concentration did not increase in the headspace and not because the fiber is saturated. It is acceptable to use different sampling parameters for testing saturation (e.g., using static sampling for saturation test, while using dynamic sampling for experiment).

Sample size

In general, the larger the sample the better. When sampling from a closed system, the equilibrium state is perturbed less from a large sample (both container and food) than from a small sample. This means that the new equilibrium state will be reached more quickly.

GC desorption depth

Desorption depth in the GC injection port only needs to be determined once for the specific GC. For optimum desorption, the center of the SPME fiber should be injected to the hottest spot in the injection port. This can be determined by using expensive accessories commercially available from GC manufacturers, or by extracting a simple standard sample and injecting the SPME fiber at different depths. The depth producing the sharpest, most intense peak is the ideal injection depth. By setting the O ring at the desired point (as shown in Figure G1.6.1), the injection depth can be maintained consistently.

Accessories

New accessories are continually made available. This unit discusses the basics necessary to conduct SPME analysis for flavor analysis. An automated sampling and injection system is available from Varian. Supelco offers a manual sampling stand setup. Injection liners are available that reduce the injection port volume to presumably produce sharper peaks. Predrilled septa for the GC are available to reduce septum coring.

Troubleshooting

No response or reduced response

A blank or nearly blank chromatogram can be the result of several situations. The concentration in the headspace may be too low for detection by GC, even with the concentrating

ability of SPME. If, however, extractions of similar samples or the same sample have previously given a response, something is likely to be wrong with the equipment or methodology. If there is no response, the fiber may be broken or the purge valve may be open during desorption. If there is a reduced response, the fiber may be too old (see Critical Parameters for discussion of fiber degradation), there may be a loss of fiber phase due to improper handling (e.g., leaving in injection port overnight, stripping through septum), there may be leaks in the sampling container, or the composition of the food sample may be inconsistent.

Highly variable response

SPME can be >95% reproducible. However, the following conditions must be carefully controlled to obtain reproducible results: sample temperature, exposure time to the headspace, sample equilibration time (if using a closed container), sample flow rate (if using a dynamic system), sample size (both food sample and container), stirring speed (if stirred), and composition of the sample.

Wide peaks

Peak width is usually reasonably sharp on the chromatogram. Peak width can sometimes be tightened by using cryogenic focusing, although this is usually unnecessary.

Anticipated Results

SPME can concentrate a compound in a headspace up to 300-fold, possibly more. It extracts and concentrates compounds in a selective manner, so that compound A may be concentrated 150 times while compound B is concentrated only 10 times during the same extraction. Only volatilized compounds are extracted by headspace SPME. Not all compounds found by solvent extraction will be extracted by headspace SPME, primarily because not all compounds volatilize. Immersion SPME gives similar results to those obtained by solvent extraction.

As an example, gas flow from a cola beverage was analyzed and calibration of benzaldehyde was performed as described in Support Protocol 1. A 100-µm-thick polydimethylsiloxane (PDMS) SPME fiber was used to extract the gas flow from the cola beverage. Since equilibrium with the flow concentration was determined to occur within 5 min for the slowest-equilibrating compound, the fiber was exposed to the gas flow for 5 min and then desorbed in the injection port of a GC/MS. A 0.01% (w/v) solution of benzaldehyde in Freon 113 was analyzed by solvent injection. Purity was found to be 99.6%, and triplicate measurements gave $A_s = 6.50 \times 10^8$. A solution of 0.1% (w/v) benzaldehyde in propylene glycol was then prepared. This solution contained other compounds being quantified, but no overlapping peaks were produced. The solution (25 ml) was stirred at 45°C with the stopcock open for at least 1 hr. Triplicate measurements gave $A_v = 4.99 \times 10^7$. The same solution was analyzed by SPME, with triplicate measurements giving $A_{SPME} = 3.00 \times 10^8$. The calibration constant for benzaldehyde using this fiber was calculated as:

$$k_{SPME} = (C_s V_s / A_s) \times (A_v / V_v) \times (1 / A_{SPME})$$
$$= (0.1 \text{ g/liter} \times 10^{-6} \text{ liter} \div 6.5 \times 10^8) \times (4.99 \times 10^7 \div 5 \times 10^{-3} \text{ liter}) \times (1/3.00 \times 10^8)$$
$$= 5.12 \times 10^{-15} \text{ g/liter/peak area}$$

The quantity of benzaldehyde in the RAS flow from cola was calculated as:

$$Q \text{ (g/liter)} = k_{SPME} \times A_{sample}$$
$$= 5.12 \times 10^{-15} \text{ g/liter/peak area} \times 3.20 \times 10^7$$
$$= 1.64 \times 10^{-7} \text{ g/liter}$$

Time Considerations

Single extraction

Once the food sample has been prepared, a single SPME analysis time depends primarily on the determined exposure time. An exposure duration of 5 min may be arbitrarily chosen. The fiber must be cleaned prior to use, taking 2 min. Longer cleaning time is necessary after extracting complex and highly concentrated samples. The GC run time is user-dependent, and usually lasts ~1 hr. The GC must cool down before the next run. During this cooling time, the SPME may be extracting another sample.

Although the number of samples that can be extracted and analyzed per day depends strongly on the sample and methods used, on the average, if one sample is processed immediately after the other, five to ten samples can be processed in an 8-hr working day.

Fiber maintenance

Fiber selection requires as many single extractions as fiber materials being evaluated (typically two to five). Fiber selection is only conducted once for extracting similar samples. Fiber conditioning must be conducted before using a commercial fiber for the first time.

Conditioning times range from 30 min to 4 hr. Fiber degradation should be monitored after approximately every ten uses (see Critical Parameters). This involves preparing a standard solution and performing a single extraction. Testing for saturation requires the preparation of a headspace with an increased compound concentration. Two single extractions are necessary. Saturation analysis is only conducted once for extracting similar samples.

Quantification for headspace extraction

Quantification or calibration of a single fiber is composed of nine GC runs. The solvent injection takes only the time required to prepare the solution. The solution in the headspace apparatus needs ≥30 min for equilibration and 5 min for extraction by SPME or with a gastight syringe. Quantification can be conducted before or after an experiment involving the SPME or when new compounds are evaluated.

Dilution analysis

Dilution analysis time depends on the number of dilutions to be conducted. A three-dilution series with duplication is composed of six SPME single samplings and GC/O runs.

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Key References

Zhang and Pawliszyn, 1993. See above.

Gives a thorough discussion of headspace SPME theory.

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Simulation of Mouth Conditions for Flavor Analysis

This unit discusses the use and design of the two mouth simulators, the retronasal aroma simulator (RAS) and the model mouth, that have successfully been verified to produce an effluent with volatile ratios similar to that found in human exhaled breath during eating. Though at a glance the apparatuses seem very different, they produce relatively similar effluents. Of obvious notability is the difference in the size of the reservoir; the RAS reservoir is 1 liter and the model mouth reservoir is 70 ml. When determining which apparatus to use, carefully consider concentration needs, absorption characteristics of compounds, and shear resistance of the food.

Figure G1.7.1 is a comparison of chromatograms from a solid-phase microextraction (SPME) from a beverage in a sealed container and from the same beverage in a mouth simulator. This comparison demonstrates that a very different volatile ratio is produced from the same food under different sampling conditions. Due to these differences, it is important to use a sampling method that simulates mouth conditions when studying flavor compositions that produce a human perception. Most methods intended to increase headspace volatile concentration, such as adding salt for salting out, do not uniformly affect volatility. For some compounds,

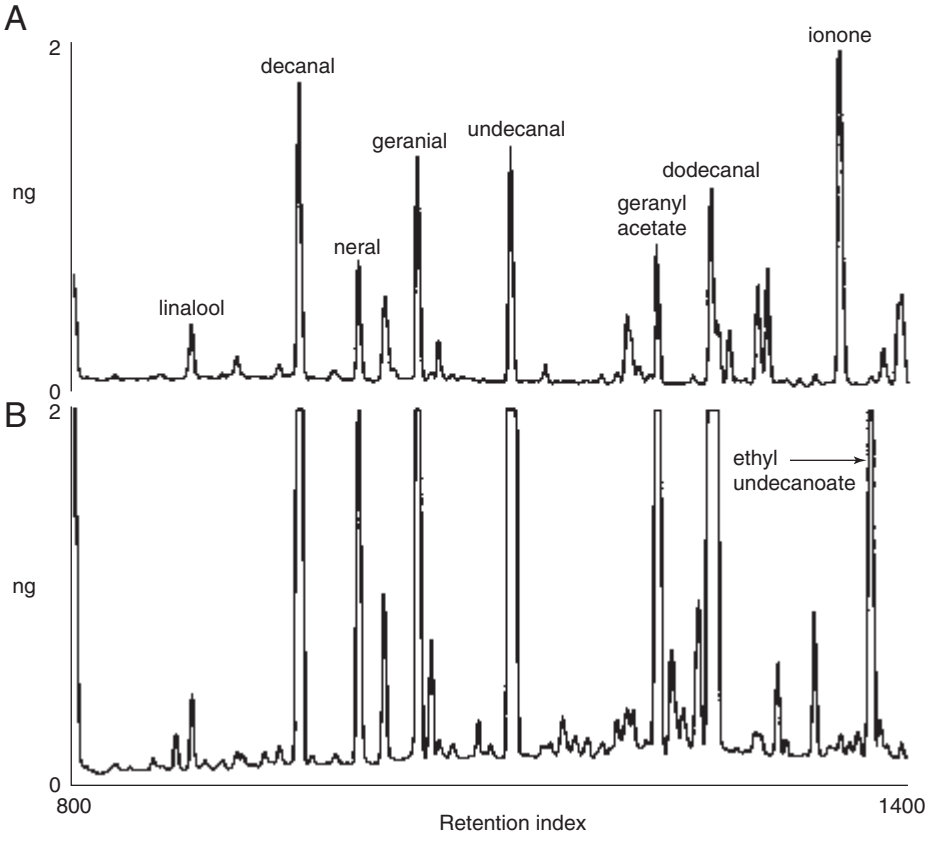

Figure G1.7.1 (**A**) A gas chromatogram of a beverage headspace sampled in the dynamic conditions of an RAS mouth simulator. (**B**) A gas chromatogram of the headspace from the same beverage under static near-equilibrium conditions. Reprinted with permission from Deibler and Acree (2000a). Copyright (2000) American Chemical Society.

Contributed by Kathryn D. Deibler and Saskia van Ruth
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vaporization increases to varying degrees; for others, it decreases to varying degrees. Combining a mouth simulator with a method to increase volatility that alters the ratio of aroma compounds in the headspace defeats the purpose of using a mouth simulator.

Direct in vivo measurement of volatiles as they escape from food in the mouth is limited primarily by sensitivity and separation by contemporary analytical equipment (Roberts, 1996). Studying the dynamic volatility from a system that simulates volatility during food consumption can help predict in vivo flavor release (Dalla Rosa et al., 1992; Bakker et al., 1996).

In the study of flavors, a mouth simulator is an apparatus designed to replicate conditions in the mouth that would affect chemical partitioning into the gas phase. This partitioning is often referred to as flavor release; however, compounds not contributing to the flavor are also affected. Ideally, a mouth simulator would create an environment that from any given food would produce the same volatile ratios as the gas phase that contacts the olfactory receptors when a person eats or drinks that food.

Most mouth simulators include features that account for temperature, breath flow, mastication, and salivation in the mouth. Temperature is usually controlled using water either in a jacket or bath. Nitrogen or purified air is blown over the food to simulate breathing. Mastication forces are created by many different means, such as a stir bar, glass balls, a plunger, or blades. Salivation is often not accounted for in mouth simulators.

Mouth simulators are part of the sample preparation process. The effluent produced from a sample in a mouth simulator may be used in conjunction with several flavor analysis methods such as CharmAnalysis (Acree et al., 1984), odor identification, and determination of detection threshold. The effluent is collected and analyzed by a variety of methods.

BASIC PROTOCOL

USE OF THE RETRONASAL AROMA SIMULATOR (RAS)

The RAS design is based on a stainless steel blender. The design is intended to be readily available, inert and easy to clean, capable of control of parameters, simple to modify for methods of headspace extraction, and, most importantly, to produce an effluent similar in ratio to the retronasal breath in humans that imparts aroma sensation (see Background Information). The RAS dimensions are such that the time to replace the gas in the reservoir per sample weight is nearly equivalent for the average human mouth. Operation, cleaning, and maintenance are rather basic. Solid or liquid food is added to the RAS, followed by artificial saliva. After the blending and air flow are started, the effluent can then be trapped on a porous polymer such as Tenax TA, sampled with SPME (*UNIT G1.6*), or directly analyzed, e.g., by MS-nose or sensory analysis (Roberts and Acree, 1995, 1996; Roberts et al., 1995, 1996; Roberts, 1996; Ong and Acree, 1998, 1999; Deibler et al., 2001, 2002).

The RAS has been verified to produce similar ratios of volatiles as produced by a human during consumption with four to sixteen panelists for over eight food types (Deibler et al., 2001). The comparisons were made by directly measuring the effluent or breath content with an MS-nose. The RAS has high precision (CV<5%) and sensitivity (µg/liter). Using large sample volumes, the odor-active volatiles can be collected and require less concentrating for the chemical analysis of trace components.

The RAS is not intended to simulate the size or structure of the mouth. The conditions in the mouth expected to affect volatility—i.e., temperature, breath flow, mastication, and salivation—are simulated. Temperature is controlled with a water jacket (37°C). Gas (N_2 or purified air) flow is controlled with a variable-area needle-valve flow meter (20 ml/sec). The shearing resulting from mastication is implemented with blender blades and a high-torque variable-

speed motor (150 rpm). Artificial saliva can be added to the system at a 1:5 (v/w) ratio of saliva to food.

The primary components of the RAS are a 1-liter stainless-steel blender container, a stainless-steel jacket that water flows through, a variable-speed motor with controller, modified lid with inlet and outlet for gas flow, and a variable-area needle-valve flow meter. The large volume allows for the collection of sufficient volatiles to concentrate trace components for GC/MS analysis. Figure G1.7.2 shows a diagram of the RAS.

Materials

Food being tested
Artificial saliva (see recipe), 37°C
Gas source (e.g., purified nitrogen, air, or humidified air)
Retronasal aroma simulator (RAS, available from DATU) with temperature-controlled water jacket or water bath

Additional reagents and equipment for headspace sampling, e.g., solid-phase microextraction (SPME, *UNIT G1.6*), porous polymer (e.g., Tenax TA trap), gas-tight syringe, MS-nose

Prepare sample

1. Bring the RAS reservoir to 37°C.
2. If the food being tested is not liquid, cut into bite-size pieces (e.g., 1.25-cm cubes of cheese).

   The RAS may not be able to break down foods with high tear resistance, such as strong gels.

3. Weigh out 150 g of food, but do not use >⅔ the volume of the RAS reservoir.

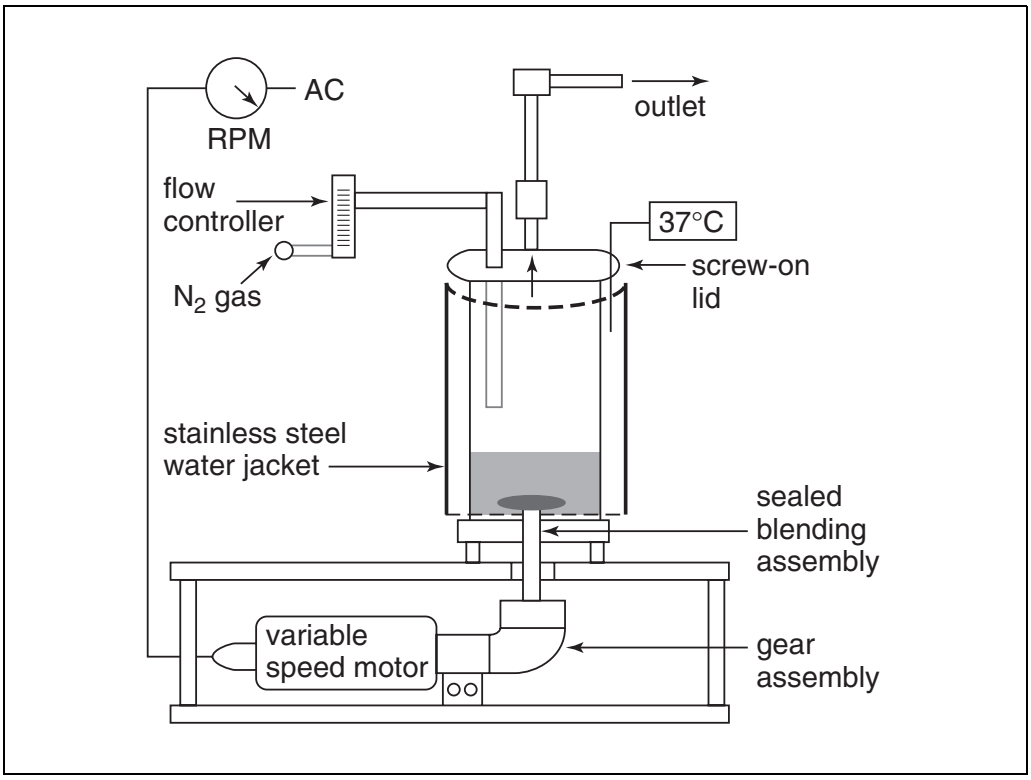

Figure G1.7.2 A diagram of the retronasal aroma simulator (RAS).

Alternatively, the amount equivalent to 30 bites or drinks may be added; then in step 5, 30 ml of artificial saliva is added (assumes food is in the mouth for 30 sec and the average simulated saliva flow rate is 2 ml/min; Bourne, 1982).

4. Add food sample to the RAS.

Analyze sample

5. Add 30 ml artificial saliva that is at ~37°C.

   More artificial saliva may need to be added to ensure that all food particles come in contact with the artificial saliva. This would be similar to an increased saliva stimulation from a dry food.

   Adding water instead of saliva may be sufficient for foods that would not be affected by the enzymes or buffering of the artificial saliva, such as soft drinks.

6. Screw lid on tightly.

7. Connect gas lines and begin flow at 20 ml/sec. Allow gas to run for ~10 sec through the system (but not through the sampling device) to purge any equilibrium or psuedo-equilibrium gas phase from the reservoir.

   The gas used may be purified nitrogen, air, or humidified air.

8. Begin blending at 150 rpm (2.5 rps).

   This produces a shear rate of ~332 sec^{-1} for a liquid, which falls within the range of shear rates in the mouth for various foods (10 to 500 sec^{-1}; Elejalde and Kokini, 1992; Roberts and Acree, 1995). Since the range of shear rates for food is large, a wide range of blending rates is acceptable (Deibler et al., 2001). If the food is not sufficiently "chewed" or broken down, a greater blending speed should be used.

   Either the blending or gas flow may be started first.

9. Begin sampling gas effluent, precisely monitoring the sampling time. Use any method of sampling headspace, including solid-phase microextraction (SPME, *UNIT G1.6*), absorption on porous polymers (e.g., Tenax TA trap), gas-tight syringe, MS-nose, and human sniffing. Multiple SPME fibers can be placed along the effluent path without depletion of the gas phase. A diversion of most of the gas flow may be necessary to avoid excessive back pressure when sampling with porous polymers. Alternatively, multiple outlets with porous polymer traps may be used.

   It is important to conduct steps 4 through 9 as quickly and reproducibly as is reasonable.

Shut down and clean RAS

10. Remove sampling device (e.g., SPME, Tenax TA trap).

11. Turn blending off.

12. Turn gas off and disconnect.

13. Evaluate collected sample, e.g., by gas chromatography/olfactometry (GC/O; Acree, 1997) or gas chromatography/mass spectrometry (GC/MS).

14. Remove from heated water jacket or disconnect reservoir from water supply.

15. Thoroughly clean and rinse reservoir and lid with soap and water.

    It may not be necessary to thoroughly dry the reservoir before its next use since water will be added to the system, provided the remaining dampness is minimal.

USE OF THE MODEL MOUTH

ALTERNATE PROTOCOL

The release of aroma compounds in the mouth during eating is primarily determined kinetically, rather than thermodynamically, because of the processes occurring when food is consumed. The model-mouth system was developed to study in vitro–like aroma release and considers the bolus volume, volume of the mouth, temperature, salivation, and mastication (van Ruth et al., 1994). Volatile compounds in the effluent of the model mouth are collected on porous polymers, such as Tenax TA. Alternatively, the effluent can be measured on-line by direct mass spectrometry techniques. The model mouth can be used to study the effects of food composition and structure on aroma release, as well as the influence of oral parameters related to eating behavior.

The model mouth is composed of a sample flask and assembly, a plunger for mastication, two voltage controllers, and two variable speed motors to give precise control of vertical and circular speed of the plunger, an externally circulating temperature-controlled water bath connected to the cavity wall of the sample flask, an externally circulating temperature-controlled ethanol bath connected to a cooling coil to freeze out water, and a controlled gas supply to sweep over the food (Figure G1.7.3).

Materials

Artificial saliva (see recipe)
Sample
Gas source (e.g., nitrogen or air)
Model mouth apparatus (Fig. G1.7.3)

1. Switch ethanol bath (B in Fig. G1.7.3) and the water bath (D) on to reach their set temperatures, −10°C and 37°C, respectively.

   The ethanol bath is used to cool a coil that will freeze out water. Water may disturb the GC analysis later. The water bath is put in place to maintain the sample flask's temperature at body temperature.

2. Place sample flask in its holder and connect the warm-water pipes.
3. Screw the cooling coil on the side of the sample flask, and connect the ethanol pipes to the coil.
4. Transfer an aliquot of artificial saliva (e.g., 4 ml) to the flask.
5. Let both water and ethanol circulate through the cavity walls of the system for 10 min to allow the system to reach the set temperatures.
6. Place one spoon of sampling material (E; e.g., 6 ml) in the sample flask.
7. Place the plunger (F) in position and connect to the motors (H).

   The plunger is the mastication device and two motors regulating the vertical and circular movements control its masticatory movements.

8. Connect a trap (A) or the connection for on-line analysis to the cooling coil.

   The gas supply (nitrogen gas or air) is connected and the flow through the trap is measured. Flow rate can be between 25 and 250 ml/min, but should be consistent for all measurements. Flow rate depends on the specific sample. For short-time analysis, flow rates are usually high to account for the dead volume. For longer measurements with traps, the breakthrough through the trap depends on the total volume passing the trap, and is thus determined by flow rate and time. For direct on-line analysis of the effluent, an atmospheric-pressure chemical ionization mass spectrometer or a proton-transfer reaction mass spectrometer is connected to the cooling coil. In case of on-line analysis, no gas supply is connected; the instrument will withdraw room air through the system at a rate determined by the instrument (15 to 300 ml/min).

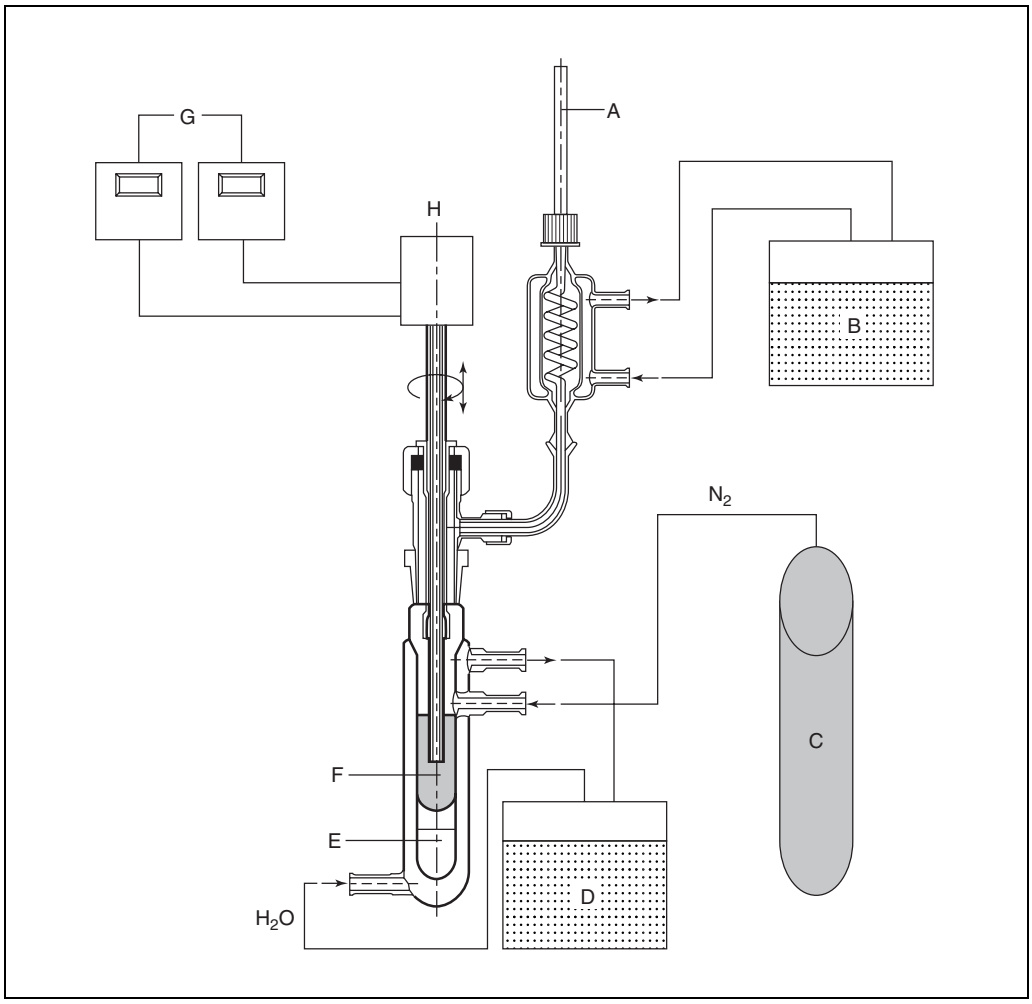

Figure G1.7.3 A diagram of the model mouth. A, trap; B, ethanol bath (–10°C); C, nitrogen gas source; D, water bath (37°C); E, sampling material (in sample flask); F, plunger; G, voltage controllers; H, motors.

9. Check the system for leaks.
10. Set the voltage controllers (G) of the variable speed motors, which regulate the vertical and circular movement of the plunger (0 to 107 cycles/min).
11. Extract the volatile compounds over a set time period. Measurement times vary between 15 sec and a few hours, and depend on the scientific requirements.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Artificial saliva

From Roth and Calmes (1981):
20 mM $NaHCO_3$
2.75 mM K_2HPO_4
12.2 mM KH_2PO_4
15 mM NaCl
200 U/ml α-amylase
Adjust pH to 7.0

From van Ruth et al. (1997):
5.208 g $NaHCO_3$
1.369 g $K_2HPO_4 \cdot 3H_2O$
0.877 g NaCl
0.477 g KCl
0.441 g $CaCl_2 \cdot 2H_2O$
0.5 g NaN_3
2.160 g mucin (porcine stomach mucin; Sigma-Aldrich)
200,000 U α-amylase (hog pancreas α-amylase; Sigma-Aldrich)
Bring to 1 liter with distilled water
Adjust to pH 7

These are two of many formulations for artificial saliva.

COMMENTARY

Background Information

Flavor perception results from interactions between a consumer and stimulants in a food. For the aroma part of flavor, the stimulants are volatiles that bind to receptor proteins found on the olfactory epithelium. These stimulants can reach the receptors by two routes, orthonasal or retronasal. The retronasal route is used when odorants are drawn from the mouth during eating through the nasal pharynx to produce aroma.

The composition of volatiles released from a food is different when it is sniffed (via orthonasal route) and when it is eaten (via retronasal route). This is partially due to conditions in the mouth that selectively affect volatility, thus altering the ratio of compounds that volatilize from a food system. Mouth temperature, salivation, mastication, and breath flow have all been shown to affect volatilization (de Roos and Wolswinkel, 1994; Roberts et al., 1994; Roberts and Acree, 1995; van Ruth et al., 1995c). The ideal gas law describes the effects of temperature. Saliva dilutes the sample, affects the pH, and may cause compositional changes through the action of the enzymes present (Burdach and Doty, 1987; Overbosch et al., 1991; Harrison, 1998). Mastication of solid foods affects volatility primarily by accelerating mass transfer out of the solid matrix. The gas flow sweeps over the food, creating a dynamic system. The rate of the gas flow determines the ratio of volatiles primarily based on individual volatilization rates and mass transfer.

The events of eating or drinking are dynamic processes in which equilibrium is never achieved (Castelain et al., 1994; de Roos, 1997; van Ruth and Roozen, 2000a). Volatiles travel to the nosespace simultaneous with mastication and drinking, as demonstrated by sensory tests using aroma solutions held in a dish in the mouth (Pierce and Halpern, 1996). In vivo measurements using a modified mass spectrometer detect significant concentration of volatiles from the nasal exhalation almost immediately after a food is placed in a person's mouth (Laing and Livermore, 1992; Linforth et al., 1994, 1999; Linforth and Taylor, 1998; Taylor et al., 2000). The concentration in the nosespace is greatly reduced at the instant the food is swallowed.

Equilibrium concentrations describe the maximum possible concentration of each compound volatilized in the nosespace. Despite the fact that the process of eating takes place under dynamic conditions, many studies of volatilization of flavor compounds are conducted under closed equilibrium conditions. Theoretical equilibrium volatility is described by Raoult's law and Henry's law; for a description of these laws, refer to a basic thermodynamics text such as McMurry and Fay (1998). Raoult's law does not describe the volatility of flavors in eating systems because it is based upon the volatility of a compound in a pure state. In real systems, a flavor compound is present at a low concentration and does not interact with itself. Henry's law is followed for real solutions of nonelectrolytes at low concentrations, and is more applicable than Raoult's law because aroma compounds are almost always present at very dilute levels (i.e., ppm). Unfortunately, Henry's law does not account for interactions with the solvent, which is common with flavors in real systems. The absence of a predictive model for real flavor release necessitates the use of empirical measurements.

Real foods are usually complex, having many components. To develop a theoretically accurate equation for a simple synthetic grape beverage containing water, sucrose, gum, and

just two aroma compounds, one would need 17 rate equations and 10 variables to define the state. Most commercial soft drinks would require >270 rate equations and 46 variables to define the state. Since rate constants are not generally available in the literature, they would all have to be individually measured. Each constant would also have to account for the viscosity and surface tension caused by the other components. Clearly, an empirical measure of the results of all these factors might be easier to obtain and be more representative. This is the role of mouth simulators.

Development of the model mouth

The model mouth was developed as part of the doctoral program of Saskia van Ruth at the Wageningen University in the Netherlands between 1992 and 1995. The hypothesis was that only those volatile compounds released under mouth conditions are relevant for aroma analysis. The volume of the mouth, temperature of the mouth, mastication, and salivation were thought to be critical parameters. Those parameters were taken into account in the first design of the instrument, which was published in 1994 (van Ruth et al., 1994). At a later stage, the design of the instrument was optimized; design changes included assemblies that allow the removal of water vapor, the technical design and construction of the plunger motor, variable speed controllers that regulate the plunger speed, and connections for on-line measurements.

The volatiles of various foods have been analyzed using the model mouth, including French beans, bell peppers, and leeks (van Ruth et al., 1995a), cheese (Lawlor et al., 2002), bulk oils and oil-in-water emulsions (van Ruth and Roozen, 2000a), and model food systems (van Ruth et al., 2000a; van Ruth and Villeneuve, in press). Factors influencing the volatile composition were studied and included post-harvesting conditions (van Ruth et al., 1995b, 1996a) and the formation of lipid oxidation products (van Ruth et al., 1999, 2000b). Oral physiological parameters examined were mastication rate (Geary et al., 2001), saliva flow rate, and saliva

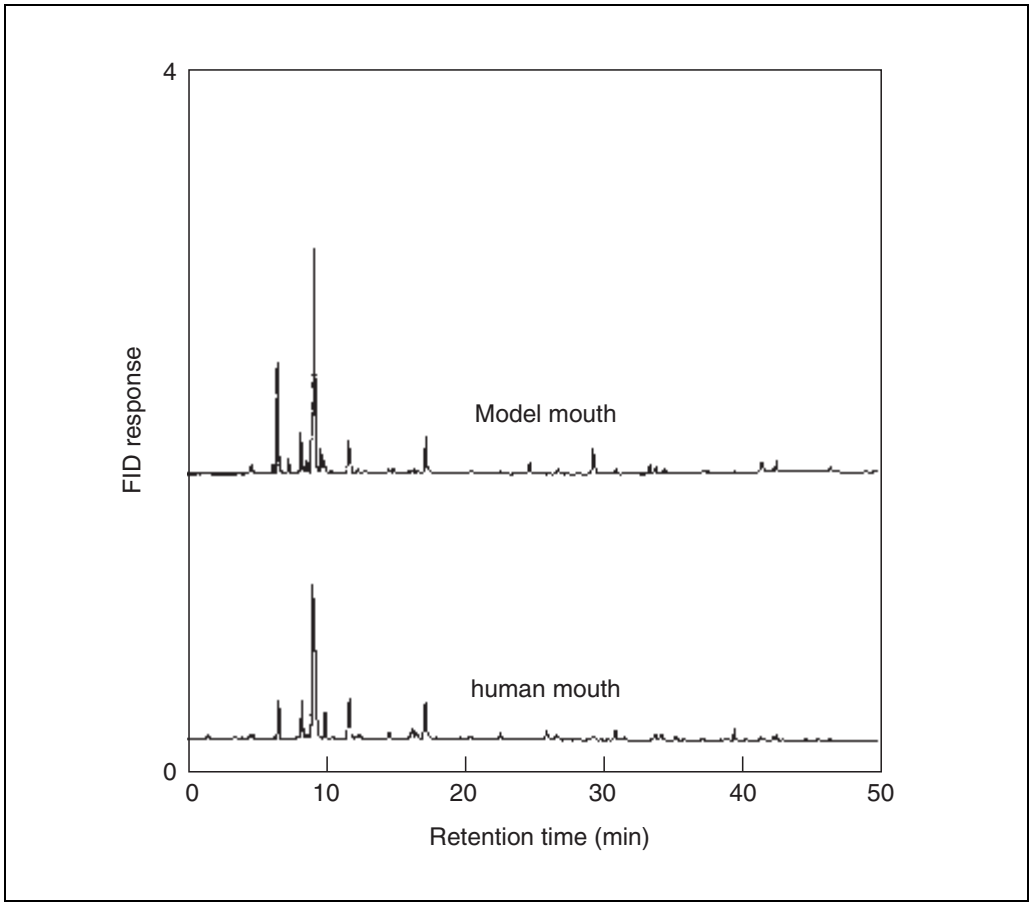

Figure G1.7.4 A model mouth and human mouth comparison shows a gas chromatogram of volatile compounds released from rehydrated French beans in the model mouth (n = 6; upper chromatogram) and in the mouth of assessors (n = 12, lower chromatogram).

composition (van Ruth et al., 1996b; van Ruth and Roozen, 2000b). Further data on the effect of oral physiological factors on volatile release can be generated with the instrument.

The model mouth was validated to be representative of volatile release in the mouth. Three dried vegetables, i.e., bell peppers, French beans, and leeks, were used for a comparison of volatile release in the model mouth and in the mouths of assessors (van Ruth et al., 1995a). An identical sample size of 1.2 g of dried vegetables and 10 ml of water were used for both techniques. In the model mouth, volatile compounds present in the effluent were extracted on the absorbent Tenax TA. In-mouth analysis was conducted during eating by withdrawing air from the mouths of twelve assessors and directing that air through a similar trap by a vacuum pump using the method described by Roozen and Legger-Huysman (1994). Gas flow rates (250 ml/min), time of isolation (12 min), chewing movements (4 movements/min), and gas chromatographic analysis of the isolated compounds were similar for model mouth and in-mouth analyses. Chromatograms of the volatile compounds extracted from the nitrogen purged through French beans in the model mouth and from oral breath are shown in Figure G1.7.4. Both qualitative and quantitative compositions of the vegetables were similar. Statistical analysis revealed no significant differences in volatile release from the three vegetables between model mouth and in-mouth analysis (Friedman two-way analysis of variance, $P < 0.05$, average CV of in-mouth 72%, of model mouth 28%). These data suggest that the model mouth mimics the volatile release in the mouth quite well.

Development of the RAS

The RAS was first designed by Roberts and Acree in 1994 using a 4-liter Waring blender (Roberts et al., 1994). The air flow rate and the blending rate were 32 ml/sec and 300 rpm, respectively. Later, a more manageable size (1-liter) Waring blender was used. This still gave the advantage of increased amount of volatiles to be concentrated. Gas flow bubbled through the liquid sample at 20 ml/sec (Roberts and Acree, 1996; Roberts et al., 1996). A chamber was added to humidify the air before it flowed through the RAS for the primary purpose of comfort for human sniffing (Deibler and Acree, 2000b). Humidification of the gas was only necessary for sensory analysis of the RAS effluent (Ong and Acree, 1998, 1999). The gas flow was redirected to no longer "purge" through the sample, but to flow over the food as in the human system (Deibler and Acree, 2000b; Deibler, 2001). Bubbling the gas through the sample would affect the ratio and quantity of volatiles released due to the different pressure found inside the bubbles. To assure that the blending rate was not affected by the resistance of the food being studied, a high-torque variable-speed motor replaced the standard blender motor. To increase efficiency of conduction of heat to the RAS reservoir, a custom-made stainless-steal jacket was used in place of the copper coils to carry water.

Sampling methods that have been used for the RAS effluent include trapping of effluent on multiple porous polymer traps with diversion of effluent when not extracting multiple or single SPME, gastight syringe extraction, MS-nose, and human sniffing. Originally, there was just one sample port for collecting volatiles from the effluent. A branched system of stainless steel tubes with multiple septa ports permitted sampling the effluent with multiple SPME units. A flow diverter allowed for the reduction of pressure through a porous polymer trap while maintaining the high gas flow rate through the RAS.

A comparison of the aroma volatiles in the RAS effluent and the aroma volatiles in nasal expiration with several foods using the MS-nose showed a close correlation (>95%; Deibler et al., 2001). Model cheese, model chocolates, and several real foods were evaluated using four to sixteen trained panelists. The variability of the RAS was <5%, while the variability between panelists was >45%, and between analyses by a single panelist was ~25%. The variability of the panelists demonstrates the vast variety of flavor release experiences existing for humans. The precision of the RAS allows for evaluation of these different experiences. Additionally, the precision of the RAS allows for controlled chemical evaluations that would be impossible with the variation of humans. The RAS produced an increased concentration (200 times), yet with nearly the same ratio of aroma compounds as human retronasal breath. This increased concentration gives greater sensitivity to analyses. Although the actual human retronasal experience is ideal for studying flavor release, this is not always practical or desirable, such as when questionable toxicity exists, when it is not cost feasible, or when the precision and sensitivity of the RAS are desired.

Table G1.7.1 Comparison of Some Mouth Simulators

	References					
Mouth parameters simulated	Lee (1986)	Roberts et al. (1994)	van Ruth et al. (1994)	de Roos and Wolswinkel (1994)	Elmore and Langley (1996)	Springett et al. (1999)
Type of air	Helium	Air or N_2	N_2	Humid N_2	Helium	N_2
Air flow (ml/min)	50	1200	20	100	30	0-120
Mastication	Shaker with balls	Blender	Plunger/screw	Stir bar	Stir bar	Stir bar
Heat	Water bath	Water coils	Water jacket	nd[a]	Water jacket	Water jacket
Salivation	Via septum	Added	Added	Could be added	Could be added	Could be added

[a]nd, not defined.

Choice of mouth simulator

The model mouth and RAS are two examples of mouth simulators. A representative set of mouth simulators are compared in Table G1.7.1. All account for temperature, breath flow, and mastication. Only the model mouth and the RAS allow for the evaluation of solid foods and have been compared directly to human breath.

The primary difference between the output from the RAS and the model mouth is concentration. Nearly equivalent volatile ratios are produced. Loss of compounds that bind to stainless steel should be considered when using the RAS. Binding to glass (e.g., by furaneol) or silanized glass (e.g., by linalool) should be considered when using the model mouth. Food with high tear resistance (e.g., some gels) may not be broken down using the RAS. This may be compensated for by using a high blending rate with additional saliva/water or by modifying the blade shape. Both mouth simulators are acceptable for most applications where a gas flow containing concentrations similar to that found in humans' retronasal breath during eating is desired.

A mouth simulator is a valuable tool when determining what volatiles contribute to the flavor sensation during consumption of a food. This includes determining potency (e.g., CharmAnalysis), intensity (e.g., OSME; Acree and Barnard, 1994), contribution (e.g., omission tests), and effect of a compound on the flavor. Sample preparation with a mouth simulator gives a close representation of the human experience, without the expense and variability of using humans. The limitations of headspace sampling and detection sensitivity define the limits of the use of mouth simulators.

The RAS has found both commercial and academic applications (Roberts and Acree, 1995; Roberts et al., 1995, 1996; Roberts, 1996; Acree, 1997; Ong and Acree, 1998, 1999; Deibler and Acree, 2000b; Deibler et al., 2000, 2001, 2002; Deibler, 2001; Feng and Acree, 2001). The effect of the mouth parameters (i.e., temperature, flow rate, shear rate, and artificial saliva) have been evaluated (Roberts, 1996; Deibler and Acree, 2000a,b; Deibler et al., 2001). The complexity of ingredient effects on flavor release from a beverage has been demonstrated (Deibler and Acree, 2000b). Individual chemical retronasal detection thresholds have been measured for wine analyses. Retronasal aroma thresholds for calculation of odor activity units have been measured using a sensory panel and the RAS (Ong and Acree, 1998, 1999). Off flavors, aroma potency, product comparison, and product formulation have been conducted both to identify and correct problems and for the successful development of several commercial products.

Critical Parameters

RAS

Of the RAS parameters, the gas flow rate has the greatest effect on volatility and must therefore be carefully controlled (Deibler et al., 2001). The flow rate of the effluent should be periodically measured to ensure flow rate consistency. The flow rate can be measured with a simple bubble meter or an electronic flow meter. Be sure the meter used is appropriate for measuring the magnitude of the anticipated flow rate.

Upon setup, on a regular periodic basis, and anytime that the flow seems compromised, the apparatus should be scanned for leaks and any

leaky connections should tightened. Leaks may be found by covering connections carrying gas with a simple soap-and-water solution or a commercial product such as Snoop Liquid Leak Detector (Nupro Company). Bubbles will be produced from any leak covered by the solution. The seals for the blade assembly and lid should be periodically tested for leaks.

If there is blockage in the gas flow path, a backpressure will develop that could potentially be dangerous. A high backpressure could cause a portion of the RAS to shoot at high speed in an unpredicted direction, potentially hurting someone. Alternatively, the backpressure could force a hole in the flow line, thus compromising the effluent being sampled. Use of a single porous polymer trap (i.e., Tenax TA) with the described flow rate will produce intolerable backpressure. This can be resolved by using a diversion flow or multiple traps.

Model mouth

Sampling time. When traps are used, the length of the time of sampling will affect the quantities of the compounds extracted. With longer extraction times, quantities will increase, but not necessarily linearly or proportionally for all compounds. As the release of aroma is a dynamic process, release rates change with time if there is significant depletion of a compound or if the release rate is zero order. The change in release rates is best observed with on-line measurements by direct mass spectrometry techniques or by interval sampling with traps. The time of extraction when traps are used is a compromise between realistic consumption times and detection limits. Breakthrough of compounds due to saturation on the porous polymer material will limit sampling time. Increasing the length and/or diameter of the sampling tube will increase the amount that the trap is able to hold, and thus increase the time that breakthrough occurs. Multiple tubes and multiple absorbent materials in series will also increase the time that breakthrough occurs. As the aim of the model mouth is to mimic mouth conditions, it is preferred to work with short isolation times (<1 min). Time of measurement should also be limited for on-line measurements.

Gas flow rates. For measurements using traps, the extraction time should be taken into account when setting the gas flow rate. With short times, gas flow rates are preferably high to account for the dead volume of the system and to reproduce in vivo breathing more accurately. If, because of detection limits (e.g., for most GC/MS), it is decided to sample for a longer time period, the breakthrough volume has to be considered. The trap will behave as an analytical column; with gas flow going through, the initially trapped compounds will move towards the end of the trap. Whether and when this happens is determined by the size of the trap, the absorbent, the volatile compound, and the total gas volume passing through the trap. The latter is determined by both the gas flow rate and time. For on-line measurements, the flow rate is usually determined by the inlet flow of the mass spectrometer. A by-pass pump may be connected to adjust the flow through the model mouth, with still the same volume going into the mass spectrometer. Again, detection limits will dictate the by-pass flow rate.

Sample size. The size of the sample will determine, to some extent, the quantity of aroma released. Generally, a larger sample size will increase the release. With relatively large samples, however, the efficiency of mastication may be affected. The obvious choice of sample size to mimic mouth conditions is the size of one bite, which varies within relatively small limits (5 to 15 g).

Sample/saliva ratio. The sample/saliva ratio is a critical parameter for volatile release in the model mouth for liquid, semi-solid, and solid foods. With liquid foods, the dilution and the change in lipid and protein concentration have an effect. With more solid types of foods, besides the effects above, saliva has an effect on the dynamics of the release. Saliva, in combination with mastication, affects the rates of mass transfer. Again, a realistic sample/saliva ratio should be chosen (e.g., in the 80:20 to 40:60 range).

Mastication rate. Mastication rates affect the extent of aroma release dramatically. A standard rate, such as 50 to 60 cycles/min, should be chosen if one is not interested in the effect of mastication rate. Usually, relatively high rates will be used (e.g., >50 cycles/min), corresponding to the chewing rates people apply when consuming solid foods. When interested in the effect of chewing behavior on flavor release, a range of mastication rates can be applied, e.g., 0, 25, 50, 75, 100 cycles/min.

Troubleshooting

RAS

Detection of no volatiles. Insufficient gas flow or insufficient blending or saliva could cause detection of no volatiles from a food with an expected high concentration of volatiles.

Reduced or absence of gas flow through the effluent outlet indicates either a leak, a blockage, or both. A blockage must be systematically discovered after any leaks have been found and repaired. If the pressure gauge (flow controller) reads the maximum reading when the flow is turned on, then the blockage is located in the system after the gauge. If the gauge registers zero while the external flow source is turned on, then the blockage or leak occurs prior to the gauge. If visual inspection does not reveal the blockage, the RAS should be taken apart starting from the outlet end, evaluating for the presence of a full-force flow after removal of each section. Once the blockage is found, it should be cleaned out and measures should be taken to prevent a blockage from re-occurring. After reassembling the RAS, check for leaks. As noted in Critical Parameters, a blockage could produce a large back-pressure that may force a part of the RAS at a high velocity in an unpredictable direction, possibly seriously injuring someone and the RAS.

If one attempts to expel the blockage using high gas/air pressure, the success depends on where and what the blockage is. The primary cause of blockage is operator error, such as a hose being clamped and forgotten, the outlet sealed by accident, or food particulates clogging the gas inlet into the RAS reservoir due to over-filling with food sample. These do not need such extreme measures as high-pressure flow to remove the blockage. Gross impurities in the gas may cause a clog. As with any equipment, the high-pressure gas should only be used on sturdy metal sections. The clogged section or valve should be removed from the system before expelling a clog with high-pressure gas. Expelling a clog with high-pressure gas is unlikely to be necessary. Teflon tubing sections not easily cleared can inexpensively and easily be replaced.

A leak can be tested for by covering gas line connections throughout the entire system with a simple soap-and-water solution or a commercial product such as Snoop Liquid Leak Detector. Bubbles will be produced from any leak covered by the solution. Tighten any leaks found and re-evaluate the flow. Some dry foods, like crackers, may require additional artificial saliva to be ground. Since dry foods often stimulate additional saliva flow, this is unlikely to compromise the flow content. Additionally, the effects of saliva have been found to have the least effect on volatility in the RAS. The shear rates resulting from mastication have a huge range (Elejalde and Kokini, 1992), thus, changing the blending rate to produce a "chewed" product may be necessary.

The detection of no volatiles from the RAS may be due to the food being tested having a concentration of volatiles below the detection limits of the sampling and analysis methods. Testing the methods with a food or model system with intense odor may indicate if the volatile concentration from the food initially being tested is just too low. A different sampling method (i.e., SPME) and/or analysis method (i.e., GC/O) may increase sensitivity enough to detect some volatiles.

Poor reproducibility. Compromised reproducibility is most likely due to something other than the RAS, such as variability of food sample, variability of the sampling method, or inconsistent application of the methodology. However, poor reproducibility can result from the RAS apparatus if there are leaks or blockages of the flow or if the unit is not properly cleaned.

Contamination in blank run. If volatiles are collected when running a blank (nothing in the RAS), there could be absorption of volatiles to the O ring that seals the lid. This can be resolved by either replacing the O ring or changing the tape that wraps the O ring. Contamination could also come from the gas source. Only pure gases should be used. Improper cleaning of the effluent sampling section is another possible source of contamination.

Saturation of sampling apparatus. Though the RAS produces an increased concentration of volatiles from foods, it is unlikely that conventional methods of sampling the effluent will cause saturation. Saturation can be indicated when a chromatogram peak is flat at the top, or when sampling of an increased concentration does not cause an increase in peak area. Changing the sampling method would be the most simple approach to eliminate saturation. Diverting some of the effluent and sampling only a fraction of the flow may eliminate saturation. Additionally, concentrations may be reduced by reducing the food sample amount; however, this change is not linear and compromises the conditions found to simulate the mouth.

Model mouth

Compounds not detected or detected in lower-than-expected concentrations. First, make sure that the problem is definitely due to a problem with the model mouth. For example, the cause of the problem may be due to the analytical equipment (e.g., gas chromatograph or mass spectrometer), inconsistencies in the food sample, and/or extraction errors. If volatile compounds are not detected or are detected in far lower-than-expected concentrations, there may be a gas leak somewhere in the system. All connections should be checked with a leak detector as described for the RAS.

If compounds are detected in lower concentrations than desirable because of detection limits and no leaks have occurred, sample size, gas flow rate, and isolation time can be adjusted to increase the total volatile quantity collected. The method can be optimized efficiently using trial runs in which one parameter is changed at a time. Be aware that volatile ratios will likely be affected by adjustments in these parameters.

Additional compounds are detected. If compounds are detected that are not expected, the purity of the gas should be checked. If the gas is not contaminated, the next step is to check the glassware and the assembly for contamination. Glassware should be cleaned with odorless detergents, rinsed with tap water three times, rinsed with distilled water three times, and dried in an odor-free drying cabinet. All water used in the model mouth should be distilled and should not have been in contact with any plastics. The artificial saliva may also be contaminated with odorous compounds. If necessary, the saliva can be prepared without the $NaHCO_3$ and can be purged with a purified nitrogen gas at a flow rate of 50 ml/min for as long as necessary. The $NaHCO_3$ can then be added and the pH adjusted afterwards. A blank run with saliva alone (i.e., no food sample) will determined if the volatile contamination has originated from the saliva. Mucin is especially known as a cause of volatile contamination.

Anticipated Results

The model mouth and the RAS will produce effluents carrying a ratio of volatile compounds that is similar to the ratio of volatiles leaving the human nose when the same food is consumed. The RAS effluent will be ~200 times more concentrated than the human breath. The RAS produces a time average representation of the retronasal breath composition.

It should be kept in mind that most analytical instruments, such as gas chromatographs and mass spectrometers, do not discriminate between volatile compounds that do or do not possess odor activity. Some form of sensory analysis must be conducted in order to select which volatile compounds contribute to the flavor of the foods. Gas chromatography-olfactometry (GC/O) is an important tool to accomplish that task.

Time Considerations

RAS

Bringing RAS to temperature requires ~20 min. To cut and measure the food takes ~5 min. Initiating RAS requires <5 min, running RAS and sampling effluent usually requires 5 to 15 min, and analyzing sample by GC/O or GC/MS takes ~1 hr. Cleaning the RAS requires ~5 min, and may be done simultaneously with analysis. The total time for GC analysis on the first sample is 1 hr and 30 min; and for subsequent samples 1 hr. The total time for direct MS analysis for the first sample is 30 min; and for subsequent samples 15 min.

Model mouth

As stated in Critical Parameters, if one is interested in the release of volatiles under mouth conditions, the time of consumption should be considered as well. In specific cases, however, detection limits may increase the time of sampling.

Bringing the model mouth to temperature requires ~20 min. Food preparation, i.e., cutting and measuring, takes ~5 min. Initiating the model mouth takes ~1 min, running the model mouth and sampling the effluent usually takes 1 min. Cleaning the model mouth requires ~10 min, and may be done concurrently with GC analysis. The time needed for GC and MS analysis is as described for the RAS.

An interesting approach is also to look into the temporal change of release of volatiles during consumption. Direct mass spectrometry techniques are able to monitor volatile concentrations in air at millisecond intervals. The time needed for GC/O, GC/MS, and direct MS analysis is as described for the RAS.

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Key References

Deibler et al., 2001. See above.

Provides a description of parameter settings for the RAS that simulate mouth conditions.

van Ruth and Roozen, 2000b. See above.

Provides a description of model mouth and some effects of oral physiological parameters.

van Ruth et al., 2000a. See above.

Provides information on the effects of volatile compound character, food matrix, and temporal release profiles.

Internet Resources

http://207.150.209.95/

Web site for DATU that provides a description of the RAS and purchasing information.

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Gas Chromatography/Olfactometry

As a bioassay, gas chromatography/olfactometry (GC/O) uses human "sniffers" to assay for odor activity among volatile analytes. The core technology used in GC/O analysis is sensory testing and psychophysical measurement. In GC/O, the complex modulation of perception caused by mixture suppression is eliminated because stimulants are experienced in isolation, combined with only purified air (Acree, 1997; van Ruth, 2001a,b). Although this greatly simplifies the perceptual issues, it also means that the results cannot be used to predict the sensory properties of mixtures without supporting sensory data (Lawless and Heyman, 1999). This unit begins with the simple direct column sniffing method (Basic Protocol 1), followed by modifications for quantification, including dilution analysis (Basic Protocol 2), time intensity (Basic Protocol 3), detection frequency (Alternate Protocol 1), and posterior-intensity (Alternate Protocol 2) methods.

NOTE: Use of human subjects requires proper documentation, even for food products. For further details, see Critical Parameters.

GAS CHROMATOGRAPHY/OLFACTOMETRY USING DIRECT SNIFFING

BASIC PROTOCOL 1

The simplest and most direct method to detect odor-active chemicals in flavors is to separate them chromatographically and to quantify them with an appropriately selective detector: the human olfactory system. Even though the odorants are present at extremely low concentrations and the sample contains many interfering compounds, the human nose will detect the odor-active components and ignore the odorless ones. Although the method of sniffing gas chromatographic effluents is more than 40 years old, modern GC/O instruments are engineered to transfer the odorants from the GC column to a purified, humidified, thermally moderated air stream without loss of resolution or interference from oxidation or background odors. Figure G1.8.1 shows an outline for a sniff port that can be built from a laboratory Venturi vacuum pump and some simple plumbing (Acree et al., 1976). The sensory data produced with the GC/O can be recorded by a variety of methods,

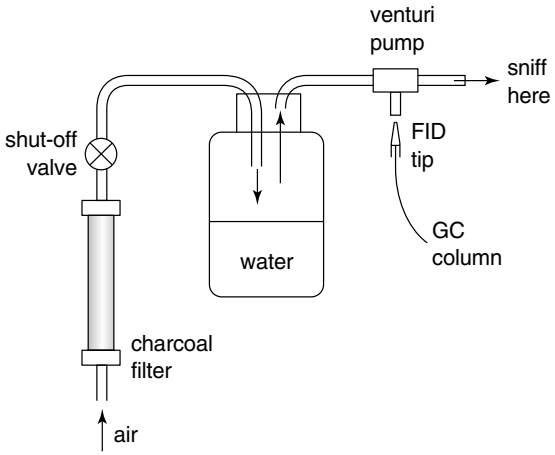

Figure G1.8.1 Diagram of the sniff port constructed from a laboratory filter (based on Acree et al., 1976; see Acree, 1997) showing the filter pump (with the check ball removed) attached to a humidifier, shut-off valve, and charcoal filter. The vacuum side of the pump is positioned over a flame ionization detector (FID) with the hydrogen gas turned off. The make-up gas helps lift the narrow (<0.2-mm-o.d.) gas chromatography (GC) effluent stream into the much larger olfactometry air stream without loss of resolution, and the 300 ml/min air combustion gas produced by the FID also prevents loss of resolution.

Contributed by Terry E. Acree and Saskia van Ruth

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from simply interrupting a strip-chart recorder to creating macros for spreadsheet programs, creating scripts for database programs, or purchasing specialized software to record both detection times and sensory perceptions. All of these methods yield tables listing the time of the onset of odor detection relative to the start of the GC program, the disappearance of odor from the olfactometer air, and a descriptor for the odor.

Materials

Indexing standards solution of *n*-alkanes: 0.005% (w/v) C_7 to C_{18} in pentane (*UNIT G1.1*)
Sniffer (i.e., human subject)
Sample to be tested (select one):
  Headspace gas or solvent extract (*UNIT G1.1*) from sample of interest
  Solid-phase microextraction (SPME) fiber, containing sample of interest (*UNIT G1.6*)
OV-101 or DB5 capillary column (e.g., 20-m × 0.32-mm; f = 0.25 µm; also see *UNIT G1.1*)
Gas chromatograph (GC; e.g., Agilent 6890, Agilent Technologies) with:
  Flame ionization detector (FID)
  Sniff port (e.g., DATU, Gerstel, Microanalytics; also see Acree et al., 1976)
  Splitless injector (linear velocity 36 cm/sec or ~2 ml/min, detector temperature, 250°C)
10-ml or 10-µl gas-tight syringe, for headspace gas or solvent extract, respectively

1. Attach an OV-101 or DB5 capillary column to the FID port of a GC and program the GC oven to run as follows:

   run isothermally for 3 min
   ramp at 2° to 6°C/min up to 225°C
   hold 10 min at 225°C.

   Most people can concentrate on sniffing for ≤20 to 30 min. The program rate can be adjusted to optimize the resolution of odors and still keep the entire run to <30 min. Rate changes need to be made at the times when a hydrocarbon (i.e., a standard) is eluting so that indexing is simple. First, a new sample should be run at 6°C/min to determine the points at which odors emerge too closely to one another. Then, at the time that the nearest standard elutes prior to the time of conflicting odors, the rate should be decreased to 2°C/min. When the next standard elutes, it should be changed back to 6°C/min. Of course, some compounds will be separated only when a different substrate is used.

2. Inject 1 µl indexing standards solution of *n*-alkanes via a splitless injector and start the GC program.

3. Record the retention time for each alkane to be used for indexing (*UNIT G1.1*).

4. Move the column from the FID to the sniff port and set up the GC for another run.

   The same GC conditions should always be used for sniffing and indexing.

5. Position a sniffer so as to provide comfortable access to the sniff port for the duration of the analysis.

   Approximately 20 min will be needed to assess effluents that range between 700 and 1800 retention index.

6. Use a 10-ml or 10-µl gas-tight syringe to inject 5 ml headspace gas or 1 µl solvent extract, or insert an SPME fiber containing the sample, and start the program. Start a timer immediately after injection.

   The sniffer should not sniff until the solvent has mostly eluted.

7. Have the sniffer breathe in constant cycles at ~20 breaths/min, record the exact moment an odor is first detected at the sniff port, associate it with a descriptor, and then record the moment the odor is no longer detectable.

   The sniffer should have been trained in advance to breathe in constant cycles and to perform the analysis.

   To automate the process, a simple macro can be written on a personal computer to record times and encode descriptors.

8. Use the data from the indexing standards to convert each time into a retention index (*UNIT G1.1*).

9. Use a database of retention indices (e.g., the Flavornet; see Internet Resource) to tentatively identify each peak.

   Verification requires sniffing an authentic standard to verify that the component and the standard have the same retention index and odor quality. Table G1.8.1 shows the result obtained when sniffing the sample used in Figure G1.8.4.

Table G1.8.1 Single Sniff Run: Start and Stop Times of Odors Detected by GC/O[a]

Start RI	Stop RI	Odor
797	800	Sweet
800	804	Sweat
831	837	Fruity
837	839	Green
839	846	Burnt sugar
882	886	Fruity
904	910	Cat urine
948	951	Minty/fruity
952	956	Mushroom
1021	1036	Cotton candy
1037	1052	Burnt sugar
1052	1072	Caramel
1072	1079	Floral
1079	1095	Fresh
1115	1123	Meat
1130	1136	Skunky
1142	1149	Sweat
1187	1195	Sweet
1216	1233	Foxy
1239	1258	Skunky
1270	1282	Foxy
1284	1307	Vanilla
1331	1347	Apple
1348	1361	Skunky/plastic
1393	1403	Plastic
1403	1413	Plastic
1419	1428	Plastic
1428	1440	Sweet
1482	1491	Cherry

[a]Data from GC/O run shown in Figure G1.8.4 (Niagara grapes; OV-101 index). The odor descriptors were the most frequent descriptors used by the sniffer over several GC/O runs of the same sample. RI, retention index.

DILUTION ANALYSIS WITH GAS CHROMATOGRAPHY/OLFACTOMETRY USING DIRECT SNIFFING

Dilution analysis uses the sniffing of sequentially diluted samples to yield quantitative measures of potency. The samples are sniffed by a direct-sniffing GC/O protocol (see Basic Protocol 1). The analyst dilutes the samples and presents them to a sniffer who records the times at which he/she smells something. Samples are usually presented in order of decreasing concentration until no odors are detected, but this is not essential (randomization to eliminate bias is possible, if needed).

Examples of GC/O dilution analysis described in the literature include CharmAnalysis (Acree, 1997) and AEDA (Aroma Extract Dilution Analysis; Acree, 1997). These methods differ in the way the data are analyzed and presented. At the end of the analysis, the lowest concentration (the highest dilution) at which an odor was detected is the flavor dilution (FD) value. Although the experimenter can write or buy software to simplify the recording and analysis, a simple graphical procedure is to set up a plot of retention index versus dilution number and draw a horizontal line that extends from the start of odor detection to its stop for each dilution tested. Simply dropping a perpendicular at the beginning or end of an area of the graph where no odor was detected will define the odor-active regions in the chromatogram. For AEDA quantification, the highest dilution detected in an odor-active region is used as the FD value. To calculate a charm value, the FD value is multiplied by the width of the odor-active region. The advantage of charm values is that they are based on peak areas and not just peak heights; this is important for a proper assessment of polar compounds, which tend to tail during chromatography.

All materials needed for dilution analysis are listed in Basic Protocol 1.

1. Prepare sequential dilutions of a liquid extract sample in the same solvent used to prepare the extract (e.g., pentane).

   It is easier to apply dilution analysis to liquid extracts than to headspace samples.

   With a 100-fold extract of grape juice diluted at 1/3, about seven or eight dilutions are required before the last odor disappears (i.e., over a 1000-fold dilution).

2. Run indexing standards and the first sample (highest concentration) as described (see Basic Protocol 1, steps 1 to 8).

3. Repeat steps 6 to 8 of Basic Protocol 1 with sequential dilutions of the sample until no odor is detected.

4. Set up a graph of RI on the abscissa versus dilution number on the ordinate and draw a horizontal line that extends from the start of an odor to its stop for each dilution tested. Drop a perpendicular at the beginning or end of an area of the graph where no odor was detected to define the odor-active regions in the chromatogram.

5. For AEDA quantification, use the highest dilution detected in an odor-active region as the FD value.

   For example:

   FD = 2^7 = 128 for the seventh dilution of a 1:1 (1/2) dilution series

   FD = 3^7 = 2187 for the seventh dilution of 1:2 (1/3) dilution series.

6. To calculate a charm value, multiply the FD value by the width (in index units) of the odor-active region.

   For example:

   charm value = 128 × 4.23 index units = 541.44 charm units.

7. Use a database of retention indices (e.g., the Flavornet; see Internet Resource) to tentatively identify each peak.

   Verification requires sniffing an authentic standard to ensure that the component and the standard have the same retention index and odor quality.

TIME INTENSITY METHOD FOR GAS CHROMATOGRAPHY/OLFACTOMETRY USING DIRECT SNIFFING

BASIC PROTOCOL 3

The time intensity method, also described as OSME (Sanchez et al., 1992), quantifies odor in terms of the psychophysical perception of intensity. It uses a cross-modal matching device (a potentiometer or a mouse on a personal computer) to instantly relate force or hand position to a perception of the changing odor intensity as stimulants rise and fall in a GC/O air stream. OSME does not measure potency (i.e., the number of times an odorant concentration is above threshold). Data are generally averaged over several sniffers to produce chromatograms with peaks that relate to perceived intensity at the sniff port. It uses the number of assessors simultaneously detecting an odor in the GC effluent (detection frequency) as a measure for the intensity of a compound. A group of assessors (six to twelve subjects) records the beginning and the end of an odor. The perceived intensities of the individual detections are combined for a specific sample to cumulate the intensity and yield a chromatogram. Data taken from a sniffing chromatogram made from the eight compounds of a reference mix for eight assessors are shown in Figure G1.8.2, where a single sniffer (HW) is shown on the left and the panel average is shown on the right. Usually, the effluent is split for two sniff ports and a flame ionization detector (FID). Thus, two assessors sniff the effluent simultaneously (without seeing each other, to avoid bias). One analysis using a group of eight assessors requires four identical gas chromatographic runs. The detection-frequency (van Ruth and Rozen, 1994; see Alternate Protocol 1) and posterior-intensity (van Ruth et al., 1996; see Alternate Protocol 2) methods of GC/O differ only in the way the data are collected and analyzed.

Materials

Reagents and equipment for GC/O with direct sniffing (see Basic Protocol 1), with additional apparatus as needed for desired number of sniffers

Cross-modal matching device: potentiometer or personal computer with mouse and in-house software for indicating perceived odor intensity

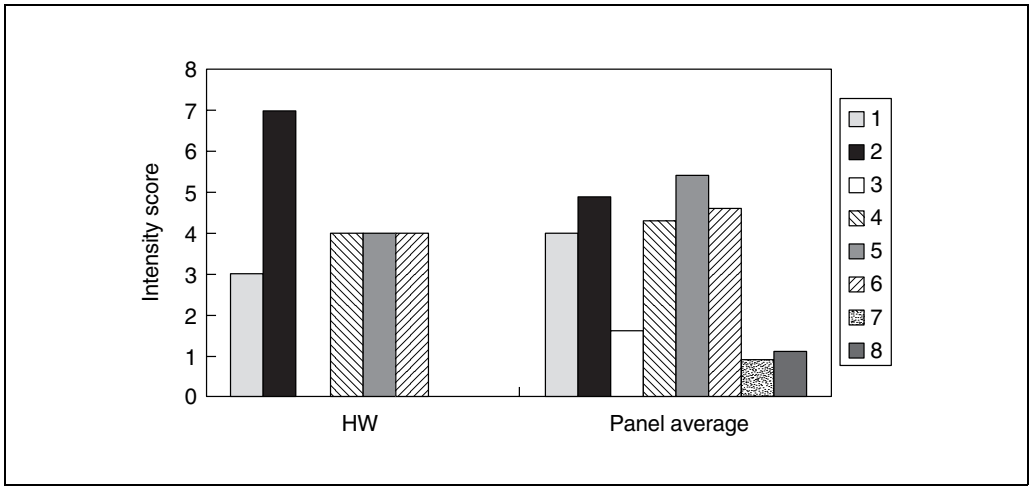

Figure G1.8.2 Time intensity chromatogram (see Basic Protocol 3), showing intensity scores of eight volatile compounds in a reference mix for a single sniffer (HW) and for the panel average (n = 8). Notice that sniffer HW showed no response to compounds 3, 7, and 8. This kind of specific anosmia is not uncommon, requiring the use of multiple sniffers or testing to eliminate anosmics.

1. Set up the necessary apparatus for the desired number of sniffers (see Basic Protocol 1, step 1). For a typical setup, split the effluent to two sniff ports and an FID so that two assessors can sniff the effluent simultaneously (without seeing each other).

   One analysis using a group of eight assessors requires four identical gas chromatographic runs.

2. Run indexing standards and inject sample as described (see Basic Protocol 1, steps 2 to 6).

3. Have the two sniffers breathe in constant cycles and record data (see Basic Protocol 1, step 7). At the same time, have the sniffers record the perceived change in intensity by using a cross-modal matching device to indicate a slide bar position or a cursor position along the length of a line.

   The software for the computer setup is not commercially available. Because published details are lacking, most users of this method write software for a personal computer to create a line (generally a thermometer widget available in higher-level languages such as Realbasic) to create a visual scale that the sniffer uses during his/her sniffing experience. The scale has been shown to be most precisely used if it is horizontal, and the ends are labeled "background" on the left and "very strong" on the right. The data generated are usually averaged and integrated to yield an average perceived intensity score (not a potency score) to quantify the group perception.

4. Repeat steps 2 and 3 for the remaining sniffers, using additional chromatographic runs.

5. Determine the intensity score of each odor for each sniffer. Determine the panel averages for each odor. Plot intensity scores of each odor.

6. Use retention indices to tentatively identify each peak (see Basic Protocol 1, steps 8 and 9).

ALTERNATE PROTOCOL 1

DETECTION FREQUENCY WITH GAS CHROMATOGRAPHY/OLFACTOMETRY USING DIRECT SNIFFING

The detection frequency method uses a number of sniffers to quantify an odor in the GC effluent from a single concentration. The underlying assumption is that any random sample of sniffers will functionally express a range of sensitivities, so that some sniffers will detect an odor and others will not. The conclusion is that the fraction of a group that detects an odor is related to the group potency of the odor, a notion that can be supported by the large diversity in odor thresholds observed in humans.

The sniffers (six to twelve subjects) record the beginning and end of detection for an odor. Data are collected in exactly the same way that they are collected during a single sniff run of Basic Protocol 1. The duration of the individual detections are combined for a specific sample to cumulate the number of detections and yield a chromatogram. An example of a sniffing chromatogram for the eight compounds of a reference mix for eight assessors is shown in Figure G1.8.3. Sometimes the effluent is split for two sniff ports and a flame ionization detector (FID). In this case, two assessors sniff the effluent simultaneously (without seeing each other, to avoid bias; see Basic Protocol 3).

The materials are the same as those listed for Basic Protocol 3.

1. Set up the necessary apparatus for the desired number of sniffers (see Basic Protocol 1, step 1). If needed, split the effluent to two sniff ports and an FID (see Basic Protocol 3, step 1).

2. Collect odor detection data as described (see Basic Protocol 1, steps 2 to 7).
3. Repeat using several (about eight) trained sniffers, with each sniffer repeating the assay about four times.

   Multiple sniffers are used to assess intensity, and repeated measures are used to reduce noise in the data.

4. Set up a graph of retention index on the abscissa versus dilution number on the ordinate and draw a horizontal line that extends from the start of an odor to its stop for each replicate tested. Drop a perpendicular at the beginning or end of an area of the graph where no odor was detected to define the odor-active regions in the chromatogram. Use the number of subjects that responded in each odor-active region as the frequency response (e.g., seven of eight subjects, for a frequency response of seven).
5. Use a database of retention indices (e.g., the Flavornet; see Internet Resource) to tentatively identify each peak.

   Verification requires sniffing an authentic standard to verify that the component and the standard have the same retention index and odor quality. Figure G1.8.3 shows a typical detection-frequency chromatogram.

POSTERIOR INTENSITY WITH GAS CHROMATOGRAPHY/OLFACTOMETRY USING DIRECT SNIFFING

ALTERNATE PROTOCOL 2

The posterior-intensity method involves the recording of the odor intensity on a scale after a peak has eluted from the column. A linear relationship has been shown to exist between the logarithm of the stimulus at the sniff port and the average posterior intensity of a panel of eight sniffers (van Ruth et al., 1996). As is frequently the case in GC/O, large variability was observed between the sniffers. The task for the sniffer is moderately complicated in the posterior-intensity method, resulting in differences in scale usage among sniffers. To overcome variation, a group of sniffers should be used and the end of the scales should be anchored with references (in theory). References can be provided only during training GC/O runs; it is practically impossible to provide a reference during an actual GC/O run. Despite good correlations between numbers of assessors and intensities at the sniff port and intensities of sensory attributes for a number of compounds, it is a drawback that the method is not based on real intensities.

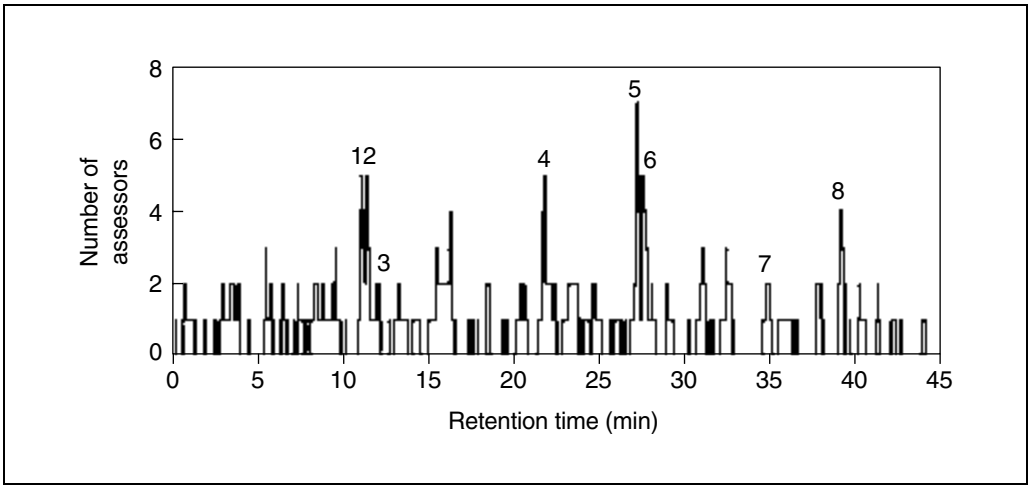

Figure G1.8.3 Sniffing chromatogram of eight volatile compounds in a reference mix obtained by the detection frequency method using eight assessors (see Alternate Protocol 1). Compounds: 1, 100 ng 2-butanone; 2, 20 ng diacetyl; 3, 500 ng ethyl acetate; 4, 100 ng 3-methyl-1-butanol; 5, 20 ng ethyl butyrate; 6, 100 ng hexanal; 7, 100 ng 2-heptanone; 8, 500 ng α-pinene.

The materials are the same as those listed for Basic Protocol 3.

1. Set up the necessary apparatus for the desired number of sniffers and collect odor detection data as described (see Basic Protocol 1, steps 1 to 7).
2. Repeat using several (about eight) trained sniffers, with each sniffer repeating the assay about four times.

   Multiple sniffers are used to assess intensity, and repeated measures are used to reduce noise in the data.

3. Set up a graph of retention index on the abscissa versus perceived intensity on the ordinate and draw a horizontal line that extends from the start of an odor to its stop for each odor detected. Drop a perpendicular at the beginning or end of an area of the graph where no odor was detected to define the odor-active regions in the chromatogram. Use the area (or just the height) of each odor-active region as the posterior intensity (PI) response (e.g., for a peak with a width of 4.35 and a height of 3, PI = $4.35 \times 3 = 13.05$ *or* PI = 3). For a group response, average the values from individual sniffers.
4. Use a database of retention indices (e.g., the Flavornet; see Internet Resource) to tentatively identify each peak.

   Verification requires sniffing an authentic standard to verify that the component and the standard have the same retention index and odor quality. Figure G1.8.4 shows a typical posterior-intensity chromatogram.

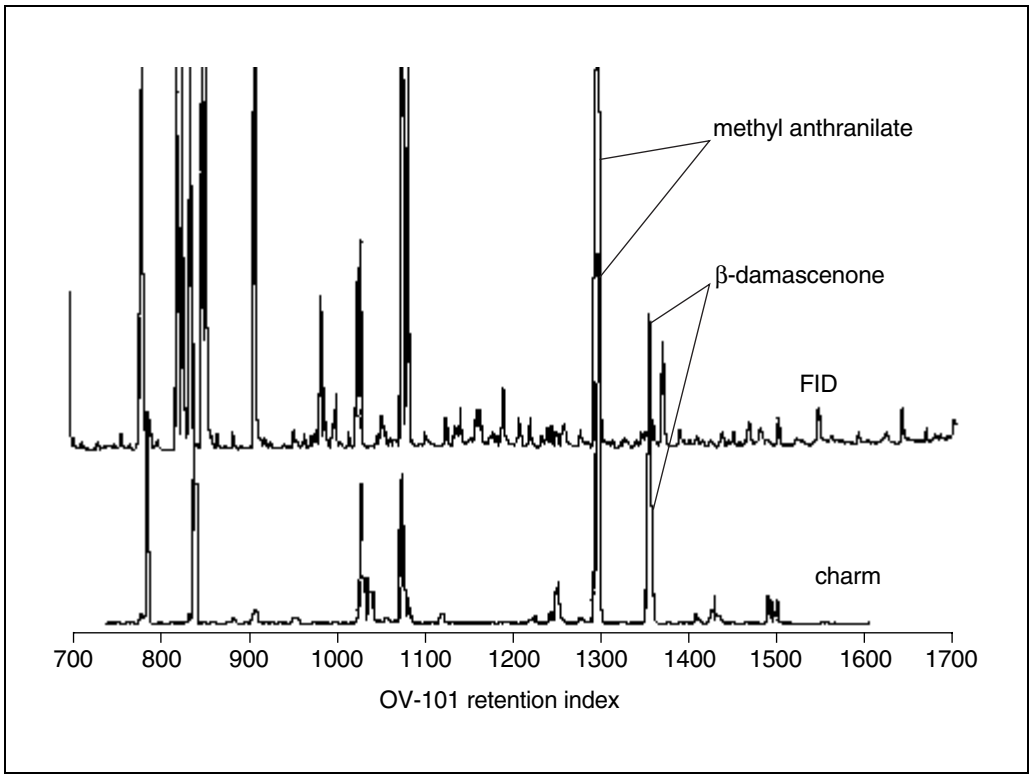

Figure G1.8.4 An FID chromatogram of concentrated extract of Niagara grape juice drawn to display the data on a linear retention index scale where the *y* axis is flame ionization response (upper trace). Below it is the charm chromatogram, where the *y* axis is dilution value. By simply comparing the index of a peak with the data listed in the Flavornet (see Internet Resource), it is possible to determine which odorants have similar retention indices. Notice how large the methyl anthranilate peak is, whereas there is no convincing peak for β-damascenone in the FID chromatogram, even though both compounds have the same potency in the charm chromatogram.

COMMENTARY

Background Information

In the early history of gas chromatography/olfactometry (GC/O; *UNIT G1.1*), the goal of GC/O analysis was to determine when an odor elutes from a GC in order to identify it. The analysis yielded a list of times and, with appropriate standards, retention indices. When combined with other chemical analysis methods, such as mass spectrometry (MS), a name for a particular odorant could be proposed. Comparing both the chemical and sensory properties of the odorant with those of authentic standards allowed researchers to identify the odorant with considerable certainty. The number of odorants that are detected, however, is determined by a number of factors, including the design of the olfactometer, the fraction of the extract injected, and, as we now suspect, the genetics of the sniffer.

Furthermore, as an extract of a natural product is concentrated, the number of odorants detected increases indefinitely. Clearly, most of the odorants in a natural product are below their odor threshold, and it is only the most potent compounds that are involved in generating the flavor response. An odorant can be very potent at extremely low concentrations if it has an extremely low odor threshold. (*UNIT G1.1*). In practice, early GC/O analysts attempted to concentrate the sample as far as possible to identify as many potential odorants as possible. Compositional studies combined with threshold studies were then used to sort out the "important" odorants from the ones that did not contribute to the flavor experience. Rothe's odor units (OU = concentration in sample/threshold in sample) were an early attempt to rank odorants by potency. The process of determining OU values for a food required a lot of chemical and psychophysical analysis. Dilution analysis was developed to produce an OU-like value directly from GC/O without the need to know the identity of the odorant. In fact, the real value of dilution analysis is that it can tell the analyst which compounds to identify.

At a meeting in Germany in 1983, the idea of using repeated sniffs of sequentially diluted samples, now generally called dilution analysis, was proposed (Acree and Barnard, 1984). This led to the publication of CharmAnalysis in 1984 and Aroma Extract Dilution Analysis (AEDA) in 1987, both of which were based on the idea of quantifying potency by dilution to threshold. Potency here is similar to the concept of titer or the amount of dilution necessary to eliminate a detectable biological response, in this case odor.

It is important to recognize that GC/O methods have no direct meaning in terms of the potency of a compound as it is experienced during eating. Extracting an odorant from a food, injecting an aliquot of the extract into a GC/O, and sniffing it as it elutes in a Gaussian distribution of concentration produced by the chromatographic process and isolated from all the other odorants, is not the same experience as eating the food. What is obtained from GC/O dilution analysis is the odor potency of each component under conditions of maximum sensitivity. By expressing the data in relative terms, the analyst hopes to prepare a priority list of the odorants in terms of their potential to contribute to the odor experience, and to avoid time wasted on the study of components that have little likelihood of contributing to the flavor experience.

Two other quantitative GC/O methods have emerged during the last 20 years: OSME and frequency response methods (e.g., GC-SNIFF). Neither of these use dilution analysis directly, but both approximate it in similar ways. Although OSME replaced potency with perceived intensity as the measured quantity, it also averaged the results of several sniffers. It is well established that humans are highly variant in their odor thresholds, and averaging the GC/O response of a number of sniffers at a single concentration will produce higher values for the most potent odorants. In a sense, the result of an OSME analysis is the average perceived intensity of an odorant at a single concentration modulated by human diversity.

The dynamic range of OSME and GC-SNIFF data is generally less than a factor of ten, whereas dilution analysis frequently yields data that cover three or four powers of ten. It has been determined, however, that compressive transforms (log, root 0.5, and so on) of dilution analysis data are needed to produce statistics with normally distributed error (Acree and Barnard, 1994). Odor Spectrum Values (OSVs) were designed to transform dilution analysis data, odor units, or any potency data into normalized values that are comparable from study to study and are appropriate for normal statistics. The OSV is determined from the equation:

$$OSV = 100 \times \sqrt{P_i / P_{\max}}$$

where P_i and P_{max} are the potency values of the ith component and the most potent component, respectively.

Critical Parameters

Exposing humans to chemicals in experimental protocols requires special procedures and documentation, even if the chemicals are extracted from foods. Human Subjects Committees must be informed of the intent to use humans in laboratory experiments, and special consent forms must be completed by each subject (referred to as a sniffer in GC/O). An example of a sniffer consent form is found in Figure G1.8.5.

Anticipated Results

Figure G1.8.4 shows a graphical display of dilution analysis data and Table G1.8.1 shows the data in tabular form. Notice that, as also shown for concord grapes in Figure G1.1.3, the methyl anthranilate peak in the FID is ten times

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Human Subjects Concerns.

1. Subjects. This research involves the testing of human response to odors they detect sniffing whole foods (cola beverages and cheeses), synthetic models of foods or extracts of foods. 1. Subjects will be trained to judge the perceived smell of flavors by sniffing them as they are separated from an instrument (a gas chromatograph-olfactometer, GCO). 2. Subjects will consist of healthy young adults recruited from Gotham City. The only criteria for exclusion will be current upper respiratory illness (colds), any known food allergies or other unusual reactions to foods, and any dietary restrictions beyond self-imposed weight control.

2. Materials. Research materials will consist of food grade flavors and natural products obtained from commercial food and flavor vendors.

3. Recruitment. Subjects will be recruited by advertisement (posters displayed in town) as well as from existing files of participants in other taste tests conducted at Gotham City. A consent form, approved by the Gotham City institutional review board, will be signed before participation, indicating the nature of the study, materials to be tasted, time involved, right to withdraw at any time without prejudice, risks, benefits and reimbursement.

4. Risks. There are few, if any, risks to the subjects. All materials will be food grade chemicals or natural products. They will be presented at dose levels equal to or less than those found in commercial foods.

5. Safeguards. All materials will be tested at levels no higher than those found in natural products, e.g., beta-damascenone occurs in apples at 100 times its threshold and will not be used at a level higher than 100 times its average reported threshold. Total intake will be limited to 1 ng per day (less than in an apple) in situations where swallowing is necessary. Rinse water and crackers will be provided to help dissipate any unpleasant residual sensations. Exposure will be limited to six sessions per day.

6. Risks/benefits. Risks to the subjects are minimal and are significantly outweighed by the scientific value of the information to be gained by the proposed studies. The only benefits to the subjects are monetary remuneration and satisfaction from participating in scientific studies.

7. Copy of the INFORMED CONSENT form to be signed by each subject that participates in the experiment is attached.

Figure G1.8.5 *(Above and at right)* Example of first and second pages of an informed consent form.

the damascenone peak, but the damascenone dilution value is the same size as for methyl anthranilate. The biggest issue for the analyst is interpretation. The simplest approach is to sort the data by charm value, FD value, OSV, and so on to see which compounds are the most potent, and then to develop compositional analyses for these compounds (*UNIT G1.6*).

Time Considerations

The biggest issue with GC/O analysis is time. It takes ~1 hr to run a blank and another hour to run a standard. With OV-101 and similar substrates, these standards need to be run only once every day or two. In this case, a complete dilution analysis of one sample could be completed in 2 days. If it takes 2 days to obtain data on one sample, the number of samples that can be analyzed is very limited, especially if replicates are analyzed for statistical comparisons. With more polar and less stable substrates, or with samples containing nonvolatile materials, the standards and blanks need to be run more often. In the worst case, the standards must be run before and after each sample. Under these conditions, a complete dilution analysis could take a week for one sample. Clearly, this is not good for any routine analysis. The results are used, however, to develop a routine GC/MS or GC/FID method to measure the most potent components.

INFORMED CONSENT

This research involves the testing of human response to odors they detect sniffing whole foods (cola beverages and cheeses), synthetic models of foods or extracts of foods. Standardized sets of odorants designed to stimulate all odor receptors in the subjects will be used in a device called a GC/O to test for sensory acuity. You will be asked to sit in front of a gas chromatograph combined with an olfactometer and sniff purified humidified air in an isolated environment. The experiment will consist of four sniffing sessions conducted on different days. The maximum number of samples you will be asked to sniff in any one day is six. Each session will take 30 to 45 minutes. You will receive ___ per hour (or any fraction of an hour) or ____ for the entire experiment for participating. If you are a student, no class credit is involved.

Your participation is strictly voluntary. You have the right to leave the experiment at any time you wish, without any penalty or hard feelings. Such a decision will not influence any other relationship that you may have to the experimenters (Jane Doe) in any way. There are no right or wrong answers in these tests. It is your unique ability to detect odors that we are interested in. After the experiment, your data will be kept in a locked file cabinet. In any electronic records, you will be identified only by a code number. Your personal data will never be displayed in any presentation or publication with your identity revealed by name or initials.

Please ask any questions you have about the study at this time.

By signing below, I indicate that I am participating in this study voluntarily. I understand that I have the right to withdraw from the experiment at any time, without penalty. I also indicate that, to the best of my knowledge, I have a normal sense of taste and smell, and that I have none of the following conditions: respiratory disease such as a cold or asthma, respiratory allergies such as hay fever, food allergies, and that I am not to the best of my knowledge pregnant or breast feeding. I have no dietary restrictions or my only dietary restrictions involve self-imposed caloric restriction for weight control. All my questions about the experiment have been answered to my satisfaction. I am between the ages of 18 and 55, inclusive.

Name (Print) _____ Date _____

Signature _____

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A general discussion of flavor analysis, including both historical material and specific applications, in which GC/O is described in the context of the most common methods of flavor analysis.

Belitz, H.-D. and Grosch, W. 1999. Food Chemistry, 2nd ed. Springer, Berlin.

An excellent summary of food analysis in which flavor chemistry is given detailed coverage with many examples.

Leland, J.V., Schieberle, P., Buettner, A., and Acree, T.E. 2001. Advances in gas chromatography-olfactometry. American Chemical Society (ACS) Symposium Series 782. ACS, Washington, D.C.

A monograph with chapters prepared by most of the leading researchers and users of GC/O in the world.

Internet Resource

http://www.nysaes.cornell.edu/flavornet

Contains retention indices on four substrates, CAS numbers, and odor qualities for >800 chemicals identified by GC/O analysis in food.

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Titratable Activity of Acid Tastants

When measuring acidity in foods and beverages there are two common units of measurement: titratable acidity and pH. There is no direct relationship between pH and titratable acidity, therefore, both must be measured experimentally. Titratable acidity (TA), also referred to as total acidity, measures the total acid content in a food or beverage system and is determined by titration of the acids in the food system with a standard base.

The term pH is used to express the concentration of free H_3O^+ in a sample and results from dissociation of the acids present. pH is defined as the negative logarithm of the hydrogen ion concentration, shown below, and can span a range of 14 orders of magnitude.

$$pH = -\log [H^+]$$

A lower pH value indicates a more acidic sample due to more free H_3O^+, and a higher pH value indicates a more basic sample. For example, lemon juice has an acidic pH (pH ~2), whereas egg whites have a basic pH (pH ~9). An accurate pH measurement is usually determined instrumentally with a pH meter.

This unit describes the methods for determining the titratable acidity in foods and beverages. To successfully determine the TA, one must have a basic understanding of pH measurements. In the Basic Protocol, titratable acidity is determined by titrating the sample with sodium hydroxide, both the potentiometric and colorimetric methods of titration are described. In order to determine the TA potentiometrically, one must know how to measure the pH of a sample. The Support Protocol describes calibrating the pH meter and pH measurement of a sample.

POTENTIOMETRIC AND COLORIMETRIC ACIDITY TITRATIONS

BASIC PROTOCOL

The choice of titration protocol to determine the TA of a sample is dependent primarily on the color of the sample. The colorimetric titration uses phenolphthalein indicator solution to determine the endpoint of the titration. Phenolphthalein indicator solution turns from colorless to pink upon reaching the endpoint; therefore, if the color of the sample interferes with this color change, the potentiometric titration is the best method. However, when using colorimetric titration, it is also common to titrate to an endpoint of pH 8.2, the endpoint of the phenolphthalein solution.

Materials

Sample
Deionized, distilled water (ddH_2O)
0.1 N sodium hydroxide solution, normalized (see recipe)
1% phenolphthalein indicator solution

pH meter (Corning, Orion)
pH electrode
250-ml beakers (potentiometric titration)
250-ml Erlenmeyer flasks (colorimetric titration)
Small magnetic stir bars
Blender (optional; if the sample needs to be macerated)
Magnetic stir plate
50-ml buret
Buret clamp
Clamp support
Stopcock
Small glass funnel

Contributed by Jane E. Friedrich
Current Protocols in Food Analytical Chemistry (2001) G2.1.1-G2.1.7

Titrate by potentiometric acidity titration

1a. Assemble apparatus as shown in Figure G2.1.1A.

2a. *Optional*: If sample is a solid, add equal parts ddH_2O and macerate in blender at 100 rpm for 2 min. If the sample is a semi-solid (i.e., fruit), macerate in blender at 100 rpm for 2 min.

If the sample is carbonated, degas prior to analysis.

3a. *Optional*: If sample contains suspended particles, carefully centrifuge for 5 min at 2500 rpm at room temperature or filter through neutral paper.

4a. Adjust the sample to room temperature or the appropriate temperature as indicated on the 10-ml volumetric pipet.

5a. Pipet 10 ml sample into a clean 250-ml beaker. Add 100 g degassed ddH_2O. Add magnetic stir bar. Cover with watch glass. Stir on magnetic stirrer until dispersed.

6a. Fill 50-ml buret with 0.1 N NaOH and slowly drain to the 0.0 starting point.

Be sure that there is no air remaining in the outlet capillary.

7a. Carefully titrate with 0.1 N NaOH solution to the endpoint of pH 8.2.

Add NaOH at a slow, uniform rate until the endpoint is approached. Then add NaOH dropwise, swirling between drops, until the endpoint is stable (e.g., 5 to 10 sec).

8a. Record buret reading for the milliliters of 0.1 N sodium hydroxide used.

9a. Calculate titratable acidity in terms of a standard acid using the equation below (see Table G2.1.1 to choose the standard acid).

$$\text{TA (g/100 ml)} = \frac{(V)(N)(\text{meq. wt.})(100)}{(1000)(v)}$$

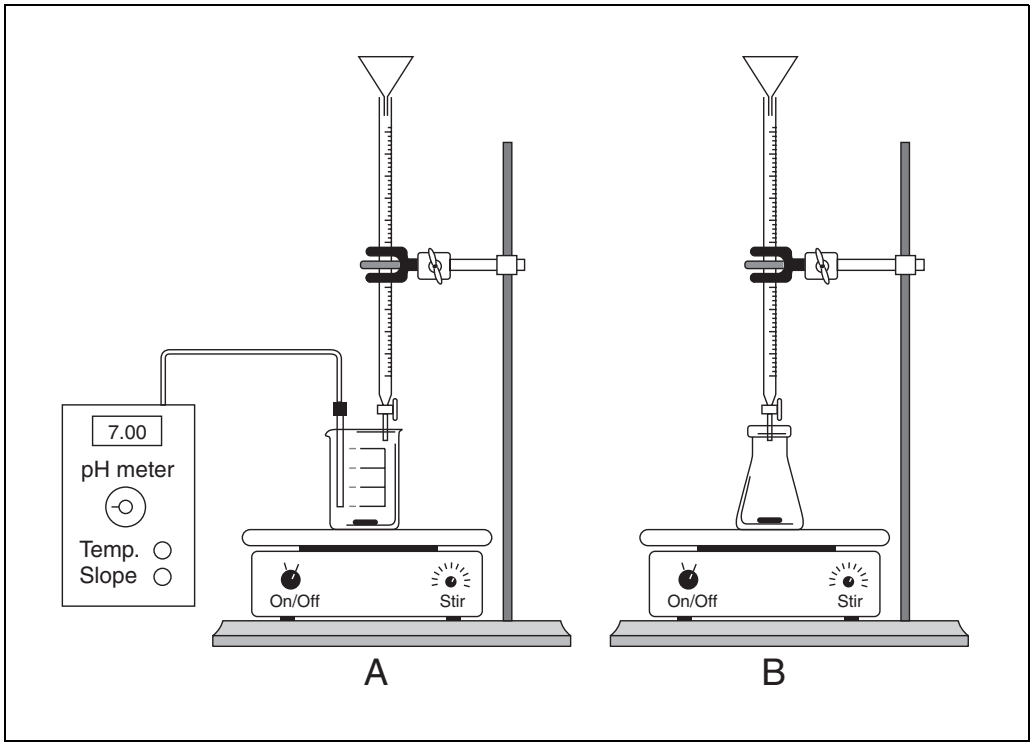

Figure G2.1.1 (**A**) Potentiometric and (**B**) colorimetric titratable acidity apparatus.

Where V is volume of sodium hydroxide solution used for titration (ml); N is normality of sodium hydroxide solution; meq. wt. is milliequivalent weight of the standard; and v is sample volume (ml).

Perform analysis in triplicate.

Titrate by colorimetric acidity titration

1b. Assemble apparatus as shown in Figure G2.1.1B.

2b. *Optional*: If sample is a solid, add equal parts ddH_2O and macerate in blender at 100 rpm for 2 min. If the sample is a semi-solid (i.e., fruit), macerate in blender at 100 rpm for 2 min.

If the sample is carbonated, degas prior to analysis.

3b. *Optional*: If sample contains suspended particles, carefully centrifuge for 5 min at 2500 rpm at room temperature or filter through neutral paper.

4b. Adjust the sample to room temperature or the appropriate temperature as indicated on the 10-ml volumetric pipet.

5b. Pipet 10 ml of sample into a clean 250-ml Erlenmeyer flask. Add 100 g degassed ddH_2O.

6b. Add 5 drops of 1% phenolphthalein indicator solution into the sample. Add magnetic stir bar and stir on magnetic stir plate.

7b. Fill 50-ml buret with 0.1 N NaOH and slowly drain to the 0.0 starting point.

Be sure that there is no air remaining in the outlet capillary.

Table G2.1.1 Standard Acids of Some Foods

Food	Standard acid[a]
Apple	Malic
Apricot	Malic
Banana	Malic
Blueberry	Citric
Cherry	Malic
Cranberry	Citric
Grapefruit	Citric
Grape	Tartaric
Lemon	Citric
Lime	Citric
Orange	Citric
Peach	Malic
Pear	Malic
Pineapple	Citric
Plum	Malic
Raspberry	Citric
Strawberry	Citric
Tomato	Citric
Wine	Tartaric

[a]meq. wt. values of the acids are: acetic acid, 60; citric acid, 64; lactic acid, 90; malic acid, 67; tartaric acid, 75; sulfuric acid, 49.

8b. Carefully titrate to the endpoint, a faint but definite pink color.

Add 0.1 N NaOH at a slow, uniform rate until the endpoint is approached. Then add NaOH dropwise until the color does not fade, i.e., the endpoint is stable (5 to 10 sec).

9b. Record buret reading for the milliliters of 0.1 N sodium hydroxide used.

10b. Calculate titratable acidity in terms of a standard acid in the sample using the equation below (see Table G2.1.1 to chose the standard acid).

$$\text{TA (g/100 ml)} = \frac{(V)(N)(\text{meq. wt.})(100)}{(1000)(v)}$$

Where V is volume (ml) of sodium hydroxide solution used for titration; N is normality of sodium hydroxide solution; meq. wt. is milliequivalent weight of the standard; v is sample volume (ml).

Perform analysis in triplicate.

SUPPORT PROTOCOL 1

pH MEASUREMENT

The pH can be measured instrumentally using a pH meter, a potentiometer, and an ion-selective electrode. The pH meter should be calibrated using buffers obtained from commercial sources. All pH meters come with model-specific instructions for calibration. The following protocol gives a basic overview of the calibration procedure and subsequent measurement for all pH meters. Review the instructions of the specific model in your laboratory to insure proper calibration.

Materials

Standard buffer solutions pH 4.00 and 7.00 (Fisher)
Deionized, distilled water (ddH_2O)
Sample

pH meter (Corning, Orion)
pH electrode
Electrode storage solution (Fisher)
25-ml beakers
50-ml beaker
Small magnetic stir bars
Magnetic stir plate
Blender (optional)
Pasteur pipet

Calibrate pH meter (2-point calibration)

1. Pour a small amount of each standard buffer solution into each of two 25-ml beaker (sufficient to cover the electrode bulb and porous plug) and place a small stir bar into each beaker.
2. Label each beaker with its pH value.
3. Allow buffers to equilibrate to room temperature.
4. Measure the temperature of the standard buffer solutions.

   This temperature should be close to the calibration temperature (indicated on the reagent bottle).

5. Adjust the temperature dial on the pH meter to the temperature of the equilibrated standard buffer solutions.

6. Set sensitivity or slope control to 100% position (ignore this step for pH meters that do not have the 100% indicated).
7. Remove the electrode from the storage solution and rinse with ddH_2O.
8. Place the pH 7.00 standard buffer solution on the magnetic stir plate and stir slowly.
9. Immerse the electrode in the pH 7.00 standard buffer solution.
10. Adjust the buffer control dial so that the display reads pH 7.00.
11. Remove the electrode from standard buffer solution and rinse with ddH_2O into a 50-ml waste beaker.
12. Place the pH 4.00 standard buffer solution on the magnetic stir plate and stir slowly.
13. Immerse the electrode in the pH 4.00 standard buffer solution.
14. Adjust the sensitivity or slope control dial so that the display reads pH 4.00.
15. Remove the electrode from standard buffer solution and rinse with ddH_2O into the 50-ml waste beaker.
16. Immerse the electrode in the pH 7.00 standard buffer solution.
17. Adjust the buffer control dial so that the display reads pH 7.00.
18. Rinse the electrode with ddH_2O into waste beaker.

Analyze sample

19. If the sample is a solid, macerate the sample in a blender at 100 rpm for 2 min.
20. Pour a small amount of the sample into three 25-ml beakers (sufficient to cover the electrode bulb and porous plug) and place a small stir bar in each.
21. Equilibrate the sample to the temperature of the standard buffer solutions.
22. Rinse the electrode with a small portion of the sample to be analyzed.

    Use a Pasteur pipet to rinse the electrode with the sample.

23. Place the beaker containing the sample on the magnetic stir plate and stir slowly.
24. Allow the dial to stabilize (i.e., 15 to 20 sec).
25. Record the pH value of the sample.
26. Repeat steps 4 through 7 to perform measurement in triplicate for each sample analyzed.
27. Once completed, rinse the electrode with ddH_2O.
28. Store the electrode in electrode storage solution.

REAGENTS AND SOLUTIONS

Use deionized, distilled water (DDH_2O) in all recipes and protocol steps.

NaOH solution, 0.1 N

Boil 1 liter ddH_2O while stirring on magnetic stir plate. Cool ddH_2O to room temperature. Place ddH_2O in beaker with magnetic stir bar on magnetic stir plate. Weigh out 4.2 g NaOH salt. Add NaOH to water while stirring on magnetic stir plate until dissolved.

Standardized NaOH solution, 0.1 N

Oven dry ~6 g of potassium acid phthalate (KHP) at 120°C for 2 hr. Cool to room temperature in dessicator. Weigh out 5 g of potassium acid phthalate in 250-ml beaker. Record exact weight. Add 100 g (100 ml) ddH_2O. Add magnetic stir bar. Cover with watch glass. Stir on magnetic stirrer until dissolved. Assemble apparatus as shown in Figure G2.1.1A. Titrate with the NaOH solution to endpoint of pH 8.2. Record ml of NaOH solution used. Calculate the normality (N) of the NaOH using the following equation:

$$N = \frac{(\text{g of KHP}) \times 1000}{(\text{ml of NaOH}) \times (\text{meq. wt. of KHP})}$$

Perform analysis in duplicate (repeat titration). Store at room temperature in a glass reagent bottle sealed with a rubber stopper. The standardized NaOH may be stored up to two months. A white swirling precipitate often appears in the standardized NaOH at the end of its shelf life, at this point the normality is still stable but this can be used as a visual sign of the end of the shelf life.

COMMENTARY

Background Information

The taste of foods and beverages often depend on the concentration and type of acid they contain. Two common concepts deal with acidity in foods: titratable acidity and pH. Each of these must be determined experimentally as each has its own impact on food quality. Over the years it has been shown that titratable acidity and pH contribute to the acid taste. However, the acid taste of food high in organic acids is dependent primarily on titratable acidity and secondarily on pH (Plane et al., 1980). The following equation has been proposed by Plane et al. to determine an acid taste index, i.e., acidity index (I_a), of a food sample:

I_a = TA (g/liter) – pH

Two methods are commonly used to determine the endpoint of an acidity titration. The potentiometric method titrates to a predetermined pH and the colorimetric method uses an indicator that changes color at a particular pH to determine the endpoint. Other methods define the endpoint as the inflection of a titration curve, i.e., plots of pH value versus milliliter of NaOH used (Sadler and Murphy, 1998; Iland et al., 1993). However, the increased precision of these methods adds little to the prediction of acid taste.

Using an endpoint of pH 8.2 as the equivalence point for potentiometric determination of TA produces results comparable to colorimetric titrations using phenolphthalein, the classic indicator for TA determinations of organic acids. Of the methods discussed here, titrating to pH 8.2 using the potentiometric titration method is the most commonly used approach and therefore the suggested method of analysis. To calculate the TA of a sample, the milliequivalent weight of a standard acid is used to expresses all of the acids present in the sample. The standard acid is often chosen to be the predominant acid in the sample (Ough and Amerine, 1988). Individual acids must be measured by more specific chemical methods, such as liquid chromatography or capillary electrophoresis.

Critical Parameters

One of the most critical parameters in the measurement of titratable acidity is the sample. The composition of sample will affect the first steps in the procedure for determining TA. For example, if the sample to be analyzed is carbonated, e.g., soft drink, beer, champagne, it

must be degassed before measuring the titratable acidity to remove the carbonic acid present in the sample. It is important to remove carbonic acid from the sample so that it will not interfere with the analysis. If the carbonic acid is not removed from the sample, the TA will be over-emphasized. High fat samples, fat content >4%, will also need to be modified prior to analysis. These samples can simply be diluted so that the total fat content is <4% prior to analysis. For further information on sample preparation, refer to Sadler and Murphy (1998). When determining TA, it is important that once the endpoint of the titration is approached, the sample must be swirled after each drop of NaOH and be allowed to reach equilibrium before further titration.

When using a pH meter, it is imperative that the meter is calibrated, and that all steps were followed to calibrate the meter, i.e., temperature was set and the slope was used to calibrate to pH 4.00. Also, the temperature of the standard buffer solutions and sample must be the same. It is recommended that the sample should be diluted prior to analysis if the sample is >15% alcohol.

Anticipated Results

The typical percent acid for the Basic Protocol will depend on the standard acid chosen. For example, if sulfuric acid is chosen as the standard acid, the percent acid will range from 0.1% to 2.4%. However, if lactic acid is chosen as the standard acid, the percent acid will range from 0.15% to 4.3% acid. Therefore, the limits of this analysis are dependent on the standard acid chosen. In general, it is hard to measure titratable acidity below 0.1% and above 4% using this method. If the titratable acidity is above 4% the sample should be diluted before analysis.

Time Considerations

The 0.1 N NaOH must be made and normalized prior to the determination of titratable acidity, ~3 hr. The first titration will take the longest due to the fact that the amount of titrant, NaOH, to be used is unknown. After the initial titration of a sample, each additional titration will take ~20 min (including calculating the titratable acidity).

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Sadler, G.D. and Murphy, P.A. 1998. pH and titratable acidity. *In* Food Analysis, 2nd ed. (S.S. Nielsen, ed.) pp. 99-118. Aspen Publishers, Inc., Gaithersburg, Md.

Key References

Sadler and Murphy, 1998. See above.

Detailed discussion of titratable acidity and pH, gives a good overview of the theory and some applications of each analysis. Also addresses sample preparation issues.

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Minneapolis, Minnesota

Liquid Chromatography of Nonvolatile Acids

Organic acids play an important role in maintaining the quality and nutritional value of a variety of foods. For example, the organic acid content of a fruit diminishes following harvest due to senescence. Organic acids also play a significant role in the overall flavor perception of foods. The sourness of cider is attributed to the content of lactic acid present in the cider. Analysis of nonvolatile acids in juices is widely used in industry for quality control and to test for adulteration. Adulteration of fruit juices is determined by looking at not only the individual organic acid concentrations but also the ratio of concentrations of the acids. The major organic acids present in cranberry juice are quinic, malic, and citric acids. Quinic acid is the most important of these acids in cranberry juice and is also the most uniform. Thus, it is often used as a marker to determine the content of cranberry juice in cranberry beverages.

Because of their importance, organic acids are one of the most commonly analyzed components of a food system. Many methods have been used to determine organic acids in foods, including volumetric, electrochemical, enzymatic, and chromatographic (paper, thin-layer, gas-liquid, or high-performance liquid chromatography) methods. Of the methods listed, high-performance liquid chromatography (HPLC) has long been used as the industry standard for the analysis of organic acids in a food sample and requires the least sample pretreatment.

This unit describes the standard method for determining the organic acids in a sample. The organic acid composition of a prepared sample can be analyzed using HPLC with a C18 column (see Basic Protocol 1) or an HPX-87H column (see Basic Protocol 2). These two methods have been shown to give comparable results. Basic Protocol 1 separates components using reversed-phase chromatography. This method uses a nonpolar stationary phase and a relatively polar mobile phase. Components will elute in order of decreasing polarity (i.e., the most polar component will elute from the column first). Basic Protocol 2 separates components using ion-exchange chromatography. Components will elute in order of increasing pKa. This method of chromatography is based on exchange equilibria between ions in solution and ions of like sign on the surface of an essentially insoluble, high-molecular-weight solid. As the two protocols use completely different mechanisms of separation, they provide complementary methods of analysis; however, it is not necessary to use both for each sample. The method of choice is dependent on the type of sample analyzed. Based on the mode of separation, Basic Protocol 1 is best when the acids expected are rather nonpolar, whereas Basic Protocol 2 would be the method of choice when there are several polar acids present. However, both methods are capable of separating a large range of organic acids. Only when questionable data (e.g., co-elution, poor peak resolution) are obtained is it necessary to apply both methods to the same sample. As both methods work fairly well for most sample types, other considerations when choosing a method would be time and cost. Basic Protocol 1 requires more time for analysis than Basic Protocol 2. The cost for columns in Basic Protocol 1 is slightly greater than in Basic Protocol 2, as only one analytical column is needed for the latter. All methods of analysis of organic acids in foods require sample pretreatment to dissolve the analytes for analysis. A Support Protocol describes methods of sample pretreatment that must be performed prior to instrumental analysis.

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BASIC PROTOCOL 1

HPLC OF NONVOLATILE ACIDS USING A C18 COLUMN

The method of high-performance liquid chromatography (HPLC) used may change depending on the organic acids present in the sample. For example, if one is analyzing the organic acids involved in the Krebs cycle in dairy products, the method of Doyon et al. (1991) has been optimized for exactly this type of analysis. However, the method described below is the most commonly used method by individuals analyzing organic acids in various foods and beverages in both academia and industry. This method is also the AOAC-tested and -approved method for determination of nonvolatile acids in apple juice and cranberry juice cocktail (Coppola and Star, 1986). This method utilizes a C18 column for HPLC analysis and has been modified to use two columns (the analytical column and a guard column) in series for the separation of organic acids (Coppola et al., 1995). The main purpose of the guard column is to increase the life of the analytical column by removing particulates and contaminants from the solvents. In this case, the guard column also helps in the analysis of products containing both tartaric and quinic acid. The use of two columns increases the resolution of the quinic acid peak.

In this procedure, the instrument is set up and standards are prepared, and then the sample is prepared as described elsewhere (see Support Protocol). Addition of an internal standard to the sample is also important for the analysis of organic acids. This provides a means not only for determining whether the analysis is working, but also for quantitating the percent recovery of the method. The sample is then run and concentrations are calculated.

Materials

0.05 M KH_2PO_4, pH 2.40 (see recipe)
HPLC-grade water
Organic acid standard solutions (e.g., see recipe) suitable for sample type
Internal standard
Sample
10% to 20% (v/v) acetonitrile (for storage of column)

High-performance liquid chromatograph (HPLC; e.g., Waters Chromatography) equipped with column heater, solvent pump, UV detector (set at 210 nm), integrator, autosampler, and (for manual injection) a 10-µl sample loop
15 × 0.46–cm YMC-ODS-AQ analytical column (AQ12S031546WT, Waters Chromatography)
YMC-ODS-AQ guard column (AQ12S05G304WTA, Waters Chromatography)
10-µl syringe (Hamilton Company, for manual injection only)

Additional reagents and equipment for sample preparation (see Support Protocol)

Set up HPLC instrument

1. Set an HPLC UV detector at 210 nm and allow to warm up for ~1 hr prior to analysis.
2. Set eluent flow rate to 1 ml/min.
3. Equilibrate a 15 × 0.46–cm YMC-ODS-AQ analytical column and a YMC-ODS-AQ guard column with mobile phase (0.05 M KH_2PO_4) for ≥30 min prior to the first injection.

   This two-column method uses a second analytical column as the guard column.

4. Obtain baseline of HPLC instrument.
5. Inject and run an HPLC-grade water sample as a blank. For manual injection, use a 10-µl syringe here and throughout procedure.

Analyze standards and samples

6. Prepare organic acid standard solutions using acids that are found in the sample.

   For each sample type, a literature review must be done prior to analysis to determine the appropriate organic acids and their concentrations. Typical acids include acetic, butyric, citric, formic, hippuric, isobutyric, isovaleric, lactic, malic, oxalic, phenylacetic, propionic, pyruvic, tartaric, uric, and valeric acids. The recipe for organic acid standard solutions (see Reagents and Solutions) describes standards that can be used for a number of fruit juices.

7. Inject standards in duplicate, record peak shapes for each acid component, and integrate results.

8. Add an internal standard to a sample. Use an acid that is not found in the sample, at a concentration that is within the range of acids that are found in the sample. Set aside an equivalent amount of internal standard in duplicate to run by itself.

   Once again, a literature review must be done to determine which acid to use as an internal standard. The internal standard will be used to calculate the percent recovery, which quantitates organic acid losses during sample preparation.

9. Prepare sample for injection (see Support Protocol).

   The sample may be prepared in advance. The internal standard should be added to the sample (step 8) before the sample is prepared (step 9) to control for losses during sample preparation.

10. Inject and run sample in duplicate. Also run internal standard alone in duplicate. Record peaks and integrate results.

11. Thoroughly flush the HPLC system and column with HPLC-grade water. For storage, flush with 10% to 20% acetonitrile to prevent microbial growth.

Calculate percent recovery

12. Determine the percent recovery of the internal standard by using the equation percent recovery = $A_{IS}/A_I \times 100$, where A_{IS} is the area of the internal standard in the sample measured in abundance units (AU), and A_I is the area of the internal standard run by itself.

Calculate acid concentrations

13. Calculate the response factor (RF) for each acid in the standard using the equation RF = C/A, where C is the concentration (ppm) of the organic acid in the standard solution and A is the peak area generated (AU).

14. Calculate the concentration of each acid in the sample using the equation $C_s = \text{RF} \times A_s$, where C_s is the concentration (ppm) of the organic acid in the sample and A_s is the area of the peak (AU) generated in the sample.

15. When analyzing drinking juices, correct for °Brix to relate the results to single-strength juice using the equation $C_{°\text{Brix}} = C_s \times (11.5/°\text{Brix of sample})$, where $C_{°\text{Brix}}$ is the concentration of an organic acid in the sample at 11.5 °Brix.

    The value 11.5 °Brix is used in the above equation as an example. The °Brix used should correspond to the juice of the product (Table G2.2.1). One needs to correct for °Brix only when reporting results as standard °Brix.

Table G2.2.1 Standard °Brix for Several Common Juices[a]

Juice	Standard °Brix
Apple	11.5
Apricot	11.7
Banana	22
Blackberry	10
Black currant	12.4
Boysenberry	10
Cranberry	7.5
Grape	16
Grapefruit	10
Lemon	7
Lime	7
Orange	11.8
Passionfruit	14
Peach	10.5
Pear	12
Pineapple	12.8
Prune	18.5
Raspberry	9.2
Sour cherry	14.7
Strawberry	8
Sweet cherry	20
Tangerine	11.8
Tomato	5

[a]From Nagy et al. (1993).

BASIC PROTOCOL 2

HPLC OF NONVOLATILE ACIDS USING AN HPX-87H COLUMN

As stated in Basic Protocol 1, the method of HPLC used may change depending on the organic acids present in the sample. This method uses a Bio-Rad Aminex HPX-87H column for HPLC analysis. An internal standard is important for the analysis of organic acids. This provides a means of not only determining if the analysis is working but also quantitating the percent recovery of the method.

Materials

0.005 M H_2SO_4 (see recipe)
HPLC-grade water
100 ppm organic acid standards (see recipe)
Internal standard
Sample
10% to 20% (v/v) acetonitrile (for storage of column)

High-performance liquid chromatograph (HPLC; e.g., Waters Chromatography) equipped with column heater, solvent pump, UV detector (set at 214 nm), integrator, autosampler, and (for manual injection) 10-µl sample loop
Aminex HPX-87H column (Bio-Rad)
Aminex HPX-87H guard column (Bio-Rad)
10-µl syringe (Hamilton Company; for manual injection only)

Additional reagents and equipment for sample preparation (see Support Protocol)

Set up HPLC instrument

1. Set an HPLC UV detector at 214 nm and allow to warm up for ~1 hr prior to analysis.
2. Set eluent flow rate to 0.6 ml/min.
3. Set column temperature to 65°C.
4. Set run time to 20 min.
5. Equilibrate an Aminex HPX-87H column and guard column with mobile phase (0.005 M H_2SO_4) for ≥30 min prior to the first injection.
6. Obtain baseline of HPLC instrument.
7. Inject and run an HPLC-grade water sample as a blank. For manual injection, use a 10-µl syringe here and throughout procedure.

Generate standard curves

8. Dilute 100 ppm organic acid standards in HPLC-grade water to prepare a series of standards ranging from 100 ppm to 10 ppm in 10 ppm increments. Use acids that are found in the sample.

   For each sample type, a literature review must be done prior to analysis to determine the appropriate organic acids and their range of concentrations for the standards. Typical acids include acetic, butyric, citric, formic, hippuric, isobutyric, isovaleric, lactic, malic, oxalic, phenylacetic, propionic, pyruvic, tartaric, uric, and valeric acids.

9. Inject each standard dilution in duplicate. Record peak shapes and integrate the results.
10. Generate a standard curve for each acid by plotting area versus concentration of organic acid for each solution. To determine the concentration of organic acid present in the sample, calculate y for the line $y = mx + b$, where y is the peak area (A) measured in abundance units (AU) produced by the organic acid in the stock solution, m is the slope of the standard curve, x is the concentration of organic acid in the solution, and b is the y intercept.

Prepare and run sample

11. Add an internal standard to a sample. Use an acid that is not found in the sample, at a concentration that is within the range of acids that are found in the sample. Set aside an equivalent amount of internal standard in duplicate to run by itself.

    Once again, a literature review must be done to determine which acid to use as an internal standard. The internal standard will be used to calculate the percent recovery, which quantitates organic acid losses during sample preparation.

12. Prepare sample for injection (see Support Protocol).

    The sample may be prepared in advance. The internal standard should be added to the sample (step 11) before the sample is prepared (step 12) to control for losses during sample preparation.

13. Inject and run sample in duplicate. Also run internal standard alone in duplicate. Record peaks and integrate results.
14. Thoroughly flush the HPLC system and column with HPLC-grade water. For storage, flush with 10% to 20% acetonitrile to prevent microbial growth.

Calculate percent recovery

15. Determine the percent recovery of the internal standard by using the equation percent recovery = $A_{IS}/A_I \times 100$, where A_{IS} is the area of the internal standard in the sample and A_I is the area of the internal standard run by itself.

Calculate acid concentrations

16. Determine the concentration of each organic acid in the sample (x in the equation from step 10) by using the sample peak area (A_s) for y.

17. When analyzing drinking juices, correct for °Brix to relate the results to single-strength juice using the equation $C_{\circ Brix} = C_s \times (11.5/°Brix \text{ of sample})$, where $C_{\circ Brix}$ is the concentration of an organic acid in the sample at 11.5 °Brix.

    The value 11.5 °Brix is used in the above equation as an example. The °Brix used should correspond to the juice of the product (Table G2.2.1). One needs to correct for °Brix only when reporting results as standard °Brix.

SUPPORT PROTOCOL

PREPARATION OF SAMPLES FOR HPLC OF ORGANIC ACIDS

All of the methods currently used to analyze organic acids in foods require some sample preparation. This is because the measurement of organic acids in a sample is actually a measurement of the analytes present. Therefore, all samples must be pretreated to dissolve the analytes for analysis. Of the various methods used to assay organic acids, HPLC calls for the least sample pretreatment on account of its high sensitivity and selectivity; this is another reason why this method is commonly used in industry. Some researchers purify samples prior to analysis to improve resolution; this is easily done with solid-liquid chromatographic techniques such as C18 Sep-Pak cartridges (*UNIT F1.1*), which will separate nonvolatile acids from anthocyanins and polyphenolics. Anion-exchange resins can be used to separate sugars from acids (*UNIT E1.2*). If the resolution of the organic acid peaks in the sample is poor, removal of the sugars, polyphenolics, and anthocyanins will improve resolution.

Materials

Liquid, semi-solid, or solid food sample
HPLC-grade water
70% to 80% (v/v) ethanol or 70% to 80% (v/v) acetonitrile (for semi-solid and solid foods only)

Refractometer
Rotary evaporator (for liquid samples <11 °Brix only)
20-ml screw-top vials
0.45-µm syringe filters (for liquid samples)
4-ml autosampler vials
Blender (for semi-solid and solid foods only)
Whatman no. 4 filter paper (for semi-solid and solid foods only)
100-ml flasks

For liquid food samples:

1a. Place a few drops of a sample on the lower prism of a refractometer and measure and record °Brix. If the sample is in the range of 11 to 12.5 °Brix, continue with step 5a. If the sample is >12.5 °Brix (i.e., it is too concentrated), continue with step 2a. If the sample is <11 °Brix, concentrate the sample using a rotary evaporator and remeasure °Brix.

    If the sample is a carbonated beverage it must first be degassed for 5 min in an ultrasonic bath prior to step 1a.

2a. Weigh ~2 g sample into a 20-ml screw-top vial.

3a. Add 10 ml HPLC-grade water, cap vial, and mix well.

4a. Measure and record °Brix. If diluted sample is still too concentrated (>12.5 °Brix), add additional water, mix well, and remeasure °Brix. If diluted sample is too dilute (<11 °Brix), add one to two drops original sample, mix well, and remeasure °Brix.

Properly diluted samples should be in the range of 11 to 12.5 °Brix.

5a. Filter sample through a 0.45-µm syringe filter into duplicate 4-ml autosampler vials and cap vials. Fill vial and discard any remaining sample.

Samples may be stored up to 24 hr at 4°C.

For semi-solid and solid food samples:

1b. Macerate ~25 g sample for 3 to 5 min with equal parts HPLC-grade water and 70% to 80% ethanol or 70% to 80% acetonitrile in a blender at 100 rpm.

The choice of ethanol or acetonitrile depends upon the type of sample; however, most samples can be analyzed using either solvent. The main difference would be that ethanol will extract the more highly polar acids better than acetonitrile.

Alternate methods of preparing aqueous extracts, especially the use of a C18 Sep-Pak cartridge, are described in UNIT F1.1.

2b. Stir slurry for 2 hr.

3b. Measure and adjust °Brix as necessary (steps 1a to 4a).

4b. Filter sample through Whatman no. 4 filter paper, washing filtrate with ~5 ml HPLC-grade water, and collect in a 100-ml flask. Fill duplicate 4-ml autosampler vials with sample, cap vials, and discard any remaining sample.

REAGENTS AND SOLUTIONS

Use HPLC-grade water in all recipes and protocol steps unless otherwise noted. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

H_2SO_4, 0.005 M

Weigh out 0.4904 g H_2SO_4 and dissolve in 1 liter water, swirling to mix. Filter through a 0.45-µm filter. Degas solution by stirring under vacuum at 635 mmHg for ≥3 hr. Store up to 1 month at room temperature (~23°C).

KH_2PO_4, 0.05 M, pH 2.40

Weigh out 6.81 g monobasic potassium phosphate and quantitatively transfer to a 1-liter beaker with water. Add additional water to ~900 ml and stir. Adjust pH to 2.40 with concentrated phosphoric acid. Quantitatively transfer solution to a 1-liter volumetric flask and bring to volume with water. Filter through a 0.45-µm filter. Store up to 1 month at room temperature (~23°C).

Organic acid standard solutions

Stock solutions: Prepare stock solutions A to E using the amounts indicated below. Measure each component to the nearest 0.1 mg and add to a 100-ml volumetric flask. Bring to volume with HPLC-grade water and mix well. Store up to 1 month at 4°C.

Stock solution A: 200 mg tartaric acid, 150 mg each acetic, isocitric, and lactic acid.

Stock solution B: 1.000 g L(–)-malic acid.

continued

Table G2.2.2 Acid Concentrations (in g/liter) in Organic Acid Standards for Selected Juices

Acid	Apple	Black currant	Grape	Grapefruit	Lemon	Orange	Pineapple
Acetic	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Citric	0.25	8.0	0.50	8.0	8.0	5.0	3.0
Galacturonic	0.20[a]						
Isocitric	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Lactic	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Malic	2.0	0.50	2.0	0.20	0.50	0.80	0.80
Quinic	0.20[a]						
Succinic	0.20[a]						
Tartaric	0.20	0.20	1.20	0.20	0.20	0.20	0.20

[a]If organic acid stock solution E is included.

Stock solution C: 1.000 g citric acid.

Stock solution D: 200 mg tartaric acid.

Stock solution E: 200 mg each galacturonic, quinic, and succinic acid.

Working standard solutions: To prepare standards, combine stock solutions A to E as appropriate for the sample. Add stock solutions to a 10-ml volumetric flask, bring to volume with HPLC-grade water, and mix well. Store up to 2 weeks at 4°C. For selected juice samples, use the amounts indicated below. For final concentrations of acids in these solutions, see Table G2.2.2.

Apple juice: 1 ml stock solution A, 2 ml stock solution B, 0.25 ml stock solution C. Addition of 1 ml stock solution E is optional.

Black currant and lemon juices: 1 ml stock solution A, 0.5 ml stock solution B, 8 ml stock solution C.

Grape juice: 1 ml stock solution A, 2 ml stock solution B, 0.5 ml stock solution C, 5 ml stock solution D.

Grapefruit juice: 1 ml stock solution A, 0.2 ml stock solution B, 8 ml stock solution C.

Orange juice: 1 ml stock solution A, 0.8 ml stock solution B, 5 ml stock solution C.

Pineapple juice: 1 ml stock solution A, 0.8 ml stock solution B, 3 ml stock solution C.

NOTE: *The standard solutions listed above are for the most common acids found in several common juices. These are not all the acids found in each juice. For example, isocitric and citric acid are commonly found in raspberry juice, and the ratio of the two is an important factor for determining adulteration of the juice.*

Organic acid standards, 100 ppm

Weigh 0.100 g each desired organic acid standard to the nearest 0.1 mg in a 10-ml volumetric flask. Bring to volume with water and mix well. Store in amber vials up to 1 month at 4°C.

COMMENTARY

Background Information

As stated earlier, organic acids play an important role in maintaining the quality and nutritional value of foods. This is because organic acids are in a constant state of flux during postharvest and tend to diminish during senescence via the Krebs cycle and the shikimic acid pathway (Haard, 1985). Organic acids are present in foods as the result of biochemical processes or from the activity of some microorganisms (mainly yeast and bacteria). They are also present due to their addition as acidulants, stabilizers, or preservatives. Organic acids possess a wide range of sensory properties. They can either play a direct role in the taste of the food, for example the sour taste of cider due to lactic acid, or have an indirect effect, as displayed by malic and acetic acid with respect to the negative correlation to sweet taste and scented flavors (Gomis and Alonso, 1996). Quinic acid is the most important of the organic acids in cranberry juice and is often used as a marker to determine cranberry juice content in cranberry beverages. Tartaric acid is regarded as a qualitative marker for the presence of grape juice, but unlike quinic acid in cranberry juice, its levels fluctuate in grape juice. Other useful qualitative markers include isocitric acid in raspberries and other berries, and the citric acid/isocitric acid ratio in berries.

Many methods have been used to determine organic acids in foods, including volumetric, electrochemical, enzymatic, and chromatographic (paper, thin-layer, gas-liquid, or HPLC) methods. However, most of these are not able to assay organic acids comprehensively; for example, the enzymatic methods are specific kits for individual organic acids (i.e., they only detect one of the acids present). Therefore, to analyze the sample comprehensively using enzymatic methods would be extremely time consuming and costly, as the analysis would have to be run separately for each acid and would require several kits.

Of the methods listed above, HPLC is the best for analyzing all organic acids in a sample, as it is able to quantitatively identify several different acids in one run. HPLC provides a method that is fast, sensitive, and reliable, which is an advantage over spectrophotometric and volumetric methods. HPLC methods are also very reproducible; the retention times of the compounds are fairly constant. However, there will be differences in retention times between columns and there will invariably be some retention time shifting on the same column over time. What is important to note is that the two different methods described in Basic Protocols 1 and 2 will give different retention times for the standards. This is because they are using different modes of separation, and as a result the retention order of the standards tends to be reversed. Of all the methods mentioned, HPLC is the most commonly used method for the analysis of organic acids.

Critical Parameters

It is imperative that the HPLC instrument, including the detector, is working correctly. The easiest way to check this is by first running a blank. If there is no response, one can move onto injecting the standards. If there is a response to the blank, the column may have been overloaded prior to this run. Refer to a troubleshooting guide for the specific HPLC system. The internet is also an invaluable source for troubleshooting (e.g., see Internet Resources). Keep in mind that the source of the problem may not be the system but may in fact be the column.

It is important to perform a comprehensive literature review prior to analysis. This is not only for the purpose of preparing standards and the addition of internal standards but also because there can be interactions between sugars and acids. For example, it has been documented that lactose can interfere with the measurement of lactic acid, which will overestimate the concentration of lactic acid in the sample. Thus, it is good to have prior knowledge of the concentrations of the acids typically found in the sample. If the resolution of the organic acid peaks is poor, it will be necessary to separate the nonvolatile acids from sugars (*UNIT E1.2*), anthocyanins, and polyphenolics.

Anticipated Results

The typical concentrations of organic acids found in samples vary greatly depending on the sample being analyzed. For example, tartaric acid is usually present in wine at 2000 to 7000 ppm, but only at 1000 to 2000 ppm in grapes, and at 75 to 200 ppm in pineapple juice. It is important to do a comprehensive literature review prior to analysis to verify that measured results are within the range of those found in the literature. For further information on the concentrations of organic acids commonly found in fruit juices, refer to Nagy et al. (1993) and Nagy and Wade (1995). Table G2.2.3 lists

Table G2.2.3 Concentrations of Organic Acids Commonly Found in Various Fruit Juices[a]

Juice	Citric acid	Isocitric acid	Malic acid	Quinic acid	Tartaric acid
Apple	0.005-0.04 g/100 g	—	0.2-1.3 g/100 g	—	—
Apricot	0.4 g/100 g	70-200 mg/kg	3.5-19 g/kg	62-76 mg/100 g	—
Cranberry	1.0 g/100 g	—	0.7 g/100 g	1 g/100 g	—
Grape	0.02-0.05 g/100 g	—	0.3-0.7 g/100 g	—	0.5-1.1 g/100 g
Grapefruit	0.9-2.0 g/100 g	—	0.02-0.1 g/100 g	—	—
Lemon	4.2 g/100 g	200 mg/liter	0.26 g/100 g	—	—
Orange	0.4-1.5 g/100 g	>44 ppm	0.1-0.3 g/100 g	—	—
Passionfruit (purple)	13.1 meq/100 g	—	13.1 meq/100 g	—	—
Passionfruit (yellow)	10.55 meq/100 g	—	10.5 meq/100 g	—	—
Pineapple	0.7-0.9 g/100 g	162 mg/liter	0.1-0.2 g/100 g	1 g/100 g	—
Raspberry	1.6 g/100 g	144 mg/liter	0.4 g/100 g	15 mg/100 g	—
Sour cherry	30 mg/100 ml	1 mg/100 ml	0.8 g/100 g	—	—
Strawberry	0.67-0.94 g/100 g	49.3 mg/kg	0.1 g/100 g	10-80 mg/100 g	—

[a]From Nagy et al. (1993) and Nagy and Wade (1995). A dash (—) indicates data not included in these reports.

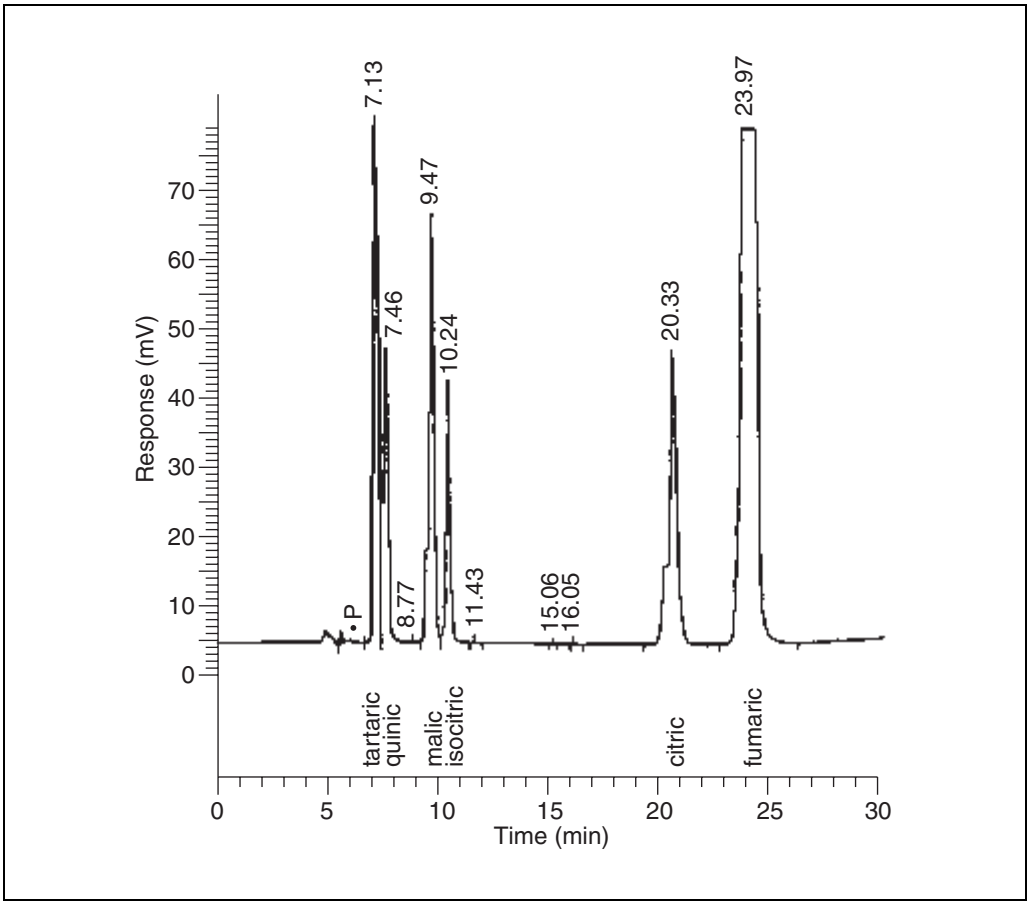

Figure G2.2.1 A typical HPLC chromatogram of organic acid standards using Basic Protocol 1. Reprinted from Coppola et al. (1995) with permission from AgScience, Inc.

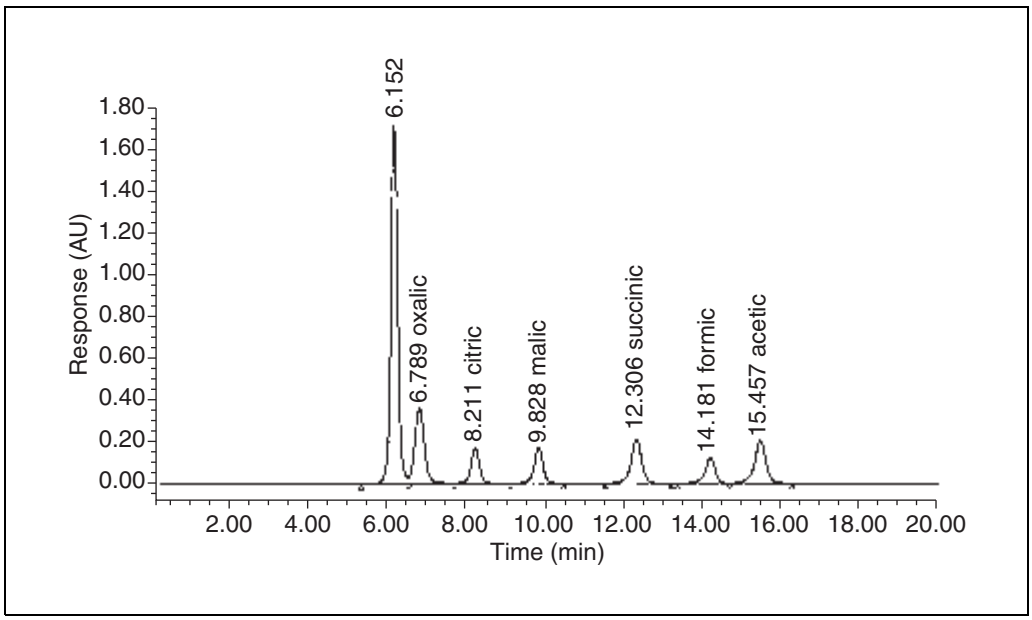

Figure G2.2.2 A typical HPLC chromatogram of organic acid standards using Basic Protocol 2.

the concentrations of organic acids commonly found in several different types of fruit juices. Figure G2.2.1 shows the separation of a standard test mix of organic acids using Basic Protocol 1. Figure G2.2.2 shows the separation of a standard test mix of organic acids using Basic Protocol 2.

Time Considerations

It will take ~2 hr to make and degas the mobile phase and prepare the standards. This must be done prior to analysis. If the sample is a semisolid or solid food, the sample pretreatment will take ~3 hr. If the sample is a liquid or beverage, the sample pretreatment will take ~1 hr. During this time it is advised to warm up the UV detector and run a blank to make sure that the HPLC unit is ready for injection of the standards.

The HPLC analysis for Basic Protocol 1 requires ~50 min per run whereas Basic Protocol 2 requires ~15 to 20 min per run. Once the standard solutions have been run for Basic Protocol 1 (~1 hr) or the standard curve has been made for Basic Protocol 2 (~2 hr), the sample can be analyzed. Sample analysis should be made in duplicate. After the HPLC analysis, the integration of the results and concentration calculations require ~15 to 30 min per organic acid.

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Internet Resources

http://www.waters.com

Includes an online troubleshooting guide.

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Overview of Viscosity and Its Characterization

WHAT IS VISCOSITY?

At this point it is appropriate to define viscosity and how it is measured. In the text that follows, the shear viscosity is referred to as viscosity. It is important to realize that fluids also have an extensional or elongational viscosity when stretched; however, this property is not dealt with here. The shear mode of deformation is assumed henceforth, such that the motion in the fluid is similar to the action of the blades of a pair of shears.

The word viscosity comes from the Latin word for mistletoe, viscum. Anyone familiar with this plant is aware that it exudes a viscous sticky sap when harvested. Viscosity is defined after Isaac Newton in his *Principia* as the ratio of stress to shear rate and is given the symbol η. Stress (σ) in a fluid is simply force/area, like pressure, and has the units of pascals (Pa; S.I. units) or dynes/cm^2 (c.g.s.). Shear rate or strain rate ($\dot{\gamma}$ or $d\gamma/dt$) is the differential of strain (γ) with respect to time. Strain is simply the change in shape of a volume of fluid as a result of an applied stress and has no units. The shear rate is in fact a velocity gradient, not a flow rate. It has the bizarre units of 1/time (sec^{-1}) and is the velocity at a given point in the fluid divided by the distance of that point from the stationary plane.

If one considers fluid flowing in a pipe, the situation is highly illustrative of the distinction between shear rate and flow rate. The flow rate is the volume of liquid discharged from the pipe over a period of time. The velocity of a Newtonian fluid in a pipe is a parabolic function of position. At the centerline the velocity is a maximum, while at the wall it is a minimum. The shear rate is effectively the slope of the parabolic function line, so it is a minimum at the centerline and a maximum at the wall. Because the shear rate in a pipe or capillary is a function of position, viscometers based around capillary flow are less useful for non-Newtonian materials. For this reason, rotational devices are often used in preference to capillary or tube viscometers.

APPARENT VISCOSITY AND SHEAR THINNING VERSUS SHEAR THICKENING

For a Newtonian fluid, viscosity is a constant at a given temperature and pressure. Viscosity is resistance to flow and is analogous to electrical resistance in a wire. The equation for viscosity correlates with Ohm's Law such that stress equals voltage, shear rate equals current, and viscosity equals resistance. Viscosity results from energy dissipation within a fluid as it is forced to flow. A fluid under laminar flow can be thought of as having many infinitesimally thin layers, called lamellae, all moving relative to one another. The friction between these layers leads to the generation of heat, and it is this phenomenon that generates fluid viscosity. Most fluids are in fact non-Newtonian, so their *apparent* viscosity (a distinction for non-Newtonian fluids) varies as a function of flow rate. This means that the energy dissipation within a fluid is either increasing (i.e., leading to an increase in viscosity) or decreasing (i.e., leading to a decrease in viscosity). The majority of non-Newtonian fluids are in fact shear thinning, in that their viscosities decrease with increasing flow. The structure of most fluids lends itself to this behavior because their components do one of the following:

1. Anisotropic particles align with the flow streamlines to reduce their hydrodynamic cross-section.
2. Aggregates of particles tend to break apart under shear forces, again minimizing hydrodynamic disturbance.
3. Polymer molecules existing as random coils elongate in the streamlines.
4. Emulsion droplets deform to become more streamlined.
5. Particles arrange themselves in formations to reduce energy, much like racing cars line up behind each other to take advantage of the leading car.

Some materials will increase in viscosity as the flow rate increases, and, although this is relatively rare, it is crucial to be aware of this if processing is to be effective. Shear thickening occurs as a result of one or more of the following situations:

1. Adjacent fluid lamellae lose their boundaries and interfere with each other, causing laminar flow to change to transitional or turbulent flow. Energy dissipation increases dramatically under these circumstances.
2. At moderate volume fractions of dispersed phase (ϕ), particles or polymers can collide or entangle, again increasing energy dissipation.

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3. Very high values of ϕ force the particles into various close-packing arrangements that can themselves be disrupted at high shear rates.

YIELD STRESS IN MATERIALS

Certain materials maintain their shape against gravity or other small stresses without flow and are therefore said to have an *apparent* yield stress. Examples of this are ketchup (the bottle must be shocked to start flow) or toothpaste (the tube must be squeezed and the extruded toothpaste maintains its cylindrical shape on the brush). It is important to remember that the lack of flow is deceptive; the viscosity is not suddenly infinite below a specific yield stress. In fact, the viscosity of such materials under a range of small stresses is simply very high (e.g., >100,000 Pa), and this means that any flow that occurs is so slow as to be undetectable over a short period of time (i.e., minutes). Over sufficiently long time scales *all* matter will flow (e.g., asphalt roads will rut, medieval lead piping will sag), so to a purist the concept of a yield stress is artifactual. To a pragmatist (e.g., a production engineer), however, if a material takes too long to flow it might just as well take forever! For this reason, the measurement of yield stress has preoccupied many industries over the last 20 years or so. It is a task that is fraught with problems because the method used to measure yield stress strongly affects the value obtained. Controlled rate devices such as laboratory viscometers will tend to give a yield stress value in proportion to the lowest shear rate they can set, while controlled stress rheometers will give much lower values because they do not force the sample to move. To this end, protocols for the measurement of yield stress are included in this chapter.

TIME-DEPENDENT BEHAVIOR

If the response of a sample to a change in shear is reversible and essentially instantaneous within the time frame of the measurement, it is said to be time independent. Most shear-thinning or shear-thickening materials are time independent. Alternatively, the sample can take seconds, minutes, hours, or longer to reach steady state; such materials are said to be time dependent. This delayed response to a change in applied shear can have a significant impact on processing considerations.

The response of time-dependent samples to reversed or looped flow measurement reveals a hysteresis. If the two steps of the flow test overlap, then the sample is able to accommodate changes of shear stress or shear rate within the time frame of the experiment. This will be the case for time-independent materials unless the ramp is so quick that inertial artifacts of the drive system are seen. In time-dependent materials, the kinetics of viscosity recovery as the shear field decreases are different from the kinetics of viscosity breakdown as the shear field is increased. Thus, the two lines no longer overlap and a thixotropic loop is seen.

The most common form of time-dependent behavior is thixotropy ("changed by touch" is the literal translation). This term used to be applied to shear-thinning properties in the paint and coatings industry, and so confusion can exist in the literature. Thixotropic materials recover structure after shearing and thus "set up" when at rest over time. This can cause problems in a manufacturing facility that has down time, because the material resting in the tanks and pipes can be difficult to pump upon startup. A very rare type of time-dependent behavior is antithixotropy or negative thixotropy. In these materials, the viscosity increases during periods of shear, and slowly declines at rest.

Thixotropy is most commonly seen in materials that are applied at high shear rates, but must then recover to a high viscosity to stay in place (e.g., nondrip paints). While such behavior is undoubtedly of value in industry, it presents significant challenges to the person responsible for characterizing the material as it is manufactured. The reason for this difficulty is the inherent sensitivity of thixotropic materials to their shear history. The effects of recent handling and differences in operator technique on the measured viscosity can be substantial. Certain flow techniques are designed specifically to allow the sample to reach steady state at each point in the test and thus have an advantage over quicker but less precise tests.

SIMPLE AND COMPLEX SHEAR

It is clear then, that the measurement of non-Newtonian materials presents special challenges for a viscometer. Many industrial viscometers designed to give a single point determination have a deceptively simple operating principle. Examples include the speed at which a liquid flows out of a container through a known orifice, a bubble rises in a column of fluid, or a ball falls in a column of fluid. These simple devices are actually very complex in terms of the shear field that is generated. The shear field is the variation of shear stress or shear rate as a function of position within the

measuring system. In measuring non-Newtonian materials, such devices will derive an average apparent viscosity giving relative data only.

To successfully measure non-Newtonian fluids, a known shear field (preferably constant) must be generated in the instrument. Generally, this situation is known as steady simple shear. This precludes the use of most single-point viscometers and leaves only rotational and capillary devices. Of these, rotational devices are most commonly used. To meet the criterion of steady simple shear, cone and plate, parallel plates, or concentric cylinders are used (Figure H1.1.1).

THE RANGE OF VISCOSITY AND SHEAR RATE

Rotational viscometers generally have a fixed range of measurement because of the limitations of their drive systems (motors). Viscometers typically generate flow viscosity data over a linear range of one to three orders of magnitude of shear rate. One specialized form of viscometer is called a rheometer. This device can measure viscoelasticity in a fluid but can also measure viscosity alone. Such devices are more expensive than viscometers, but can measure viscosity over wider ranges of shear rate or shear stress. This means that the processing engineer or formulator has access to more information.

The viscosity of non-Newtonian materials can vary by many orders of magnitude, and it is important to know as much of this range as possible. Differences in food stability can be seen at ultra-low shear rates (<0.01 sec^{-1}), while differences in consumption are seen at moderate shear rates (~50 sec^{-1}), and differences in application of the product (e.g., spreading peanut butter) are seen at high shear rates (>100 sec^{-1}).

A rheometer generates more information about a material over much wider ranges of

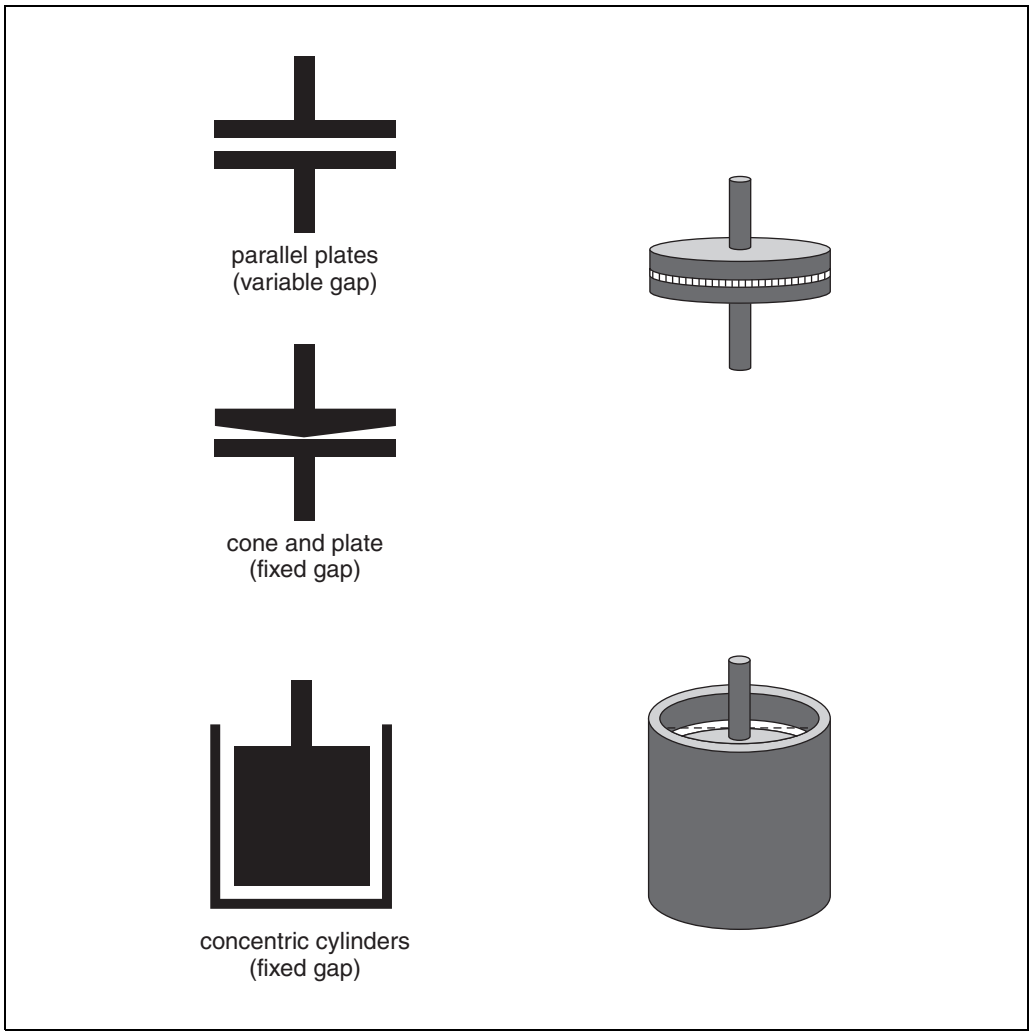

Figure H1.1.1 Common fixtures used for viscosity measurements with rotational rheometers. Cross-sectional views are shown on the left and external views are shown on the right.

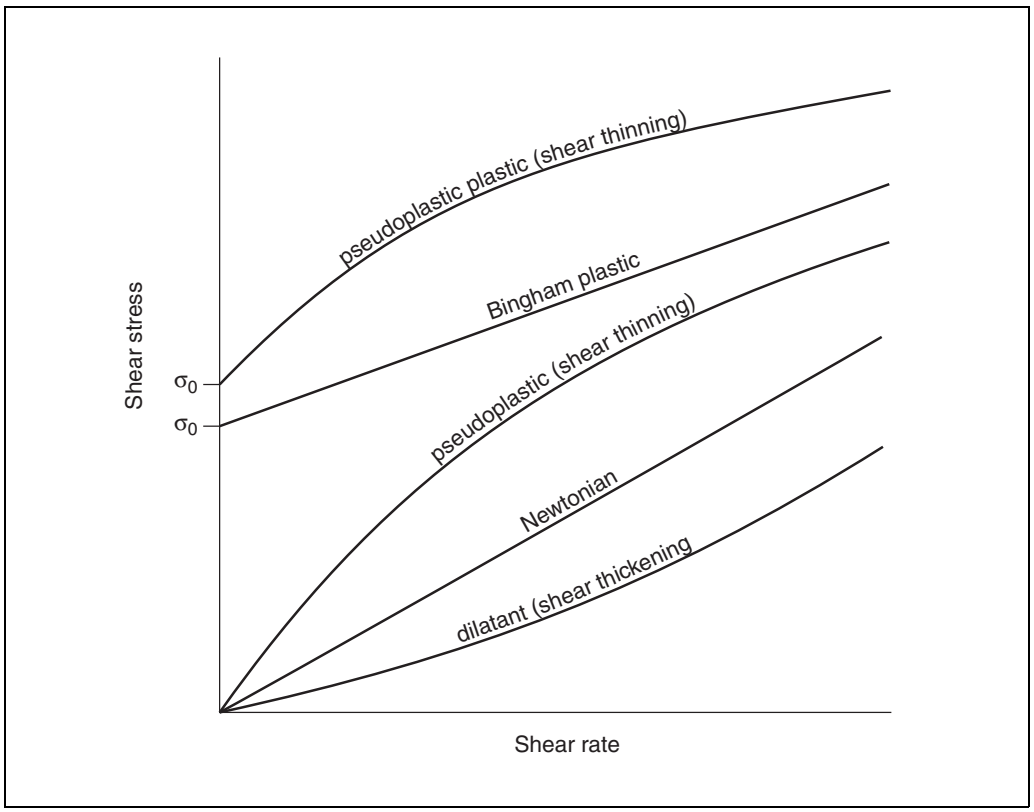

Figure H1.1.2 Rheograms or flow curves for five time-independent fluids. The Newtonian fluid yields a straight line that emanates from the origin. The other four examples are non-Newtonian fluids. σ_0 represents a yield stress point, which is common for plastic fluids.

shear rate than a viscometer. In that sense, a viscometer is to a rheometer as a hand lens is to a microscope. Certain types of rheometers can cover ranges of shear rate of six to nine orders of magnitude.

RHEOMETERS: CONTROLLED RATE AND CONTROLLED STRESS

The drive system of a rotational rheometer is likely to be optimized in one of two ways depending upon its preferred mode of operation. The most common form of rheometer is a controlled-rate (controlled-speed) device. This configuration is also used in most viscometers and has been around for decades. A shear rate is applied to a rotor by the motor controlling the viscometer's speed. The rotor is normally a flat plate or cylindrical cup. The stator is thus a cone or plate for the first two geometries or a cylindrical bob for the third (Figure H1.1.1). The stator is linked to the rotor via the sample, which acts to couple the input signal like an automobile transmission. Thus, the torque on the stator when measured by a transducer is used to derive the shear stress in the sample.

In the second type of rheometer, the controlled-stress device, the motor is optimized to apply a torque to the rotor by an induction motor and measure its resultant displacement or speed by an optical encoder. The geometry dimensions allow for the stress to be derived from the applied torque, while the gap allows for the shear rate to be derived from the displacement or speed. These novel rheometers are growing very rapidly in popularity from their introduction in the mid-1980s, owing to their ability to probe the behavior of a sample in the region where it yields or begins to flow. By applying a stress the sample can respond naturally without being forced to flow (an inevitable consequence of setting a shear rate).

VISCOMETER DATA: THE RHEOGRAM

Data from viscometers are often presented as a linear plot of shear stress versus shear rate, sometimes called a rheogram (Figure H1.1.2). This type of plot allows the viewer to see directly if there is Newtonian behavior because the plot will take the form of a straight line through the origin. A non-Newtonian response is, by definition, nonlinear and may or may not pass through the origin. If the sample has an apparent yield stress, then the line or curve will

Newtonian	$\sigma = \eta\,\dot{\gamma}$	
Pseudoplastic	$\sigma = k\,\dot{\gamma}^n$	$n < 1$
Dilatant	$\sigma = k\,\dot{\gamma}^n$	$n > 1$
Bingham	$\sigma = \sigma_0 + \eta_{PL}\dot{\gamma}$	
Casson	$\sigma^{1/2} = \sigma_0^{1/2} + \eta_c^{1/2}\dot{\gamma}^{1/2}$	
Herschel-Bulkley	$\sigma = \sigma_0 + k\,\dot{\gamma}^n$	

Figure H1.1.3 Empirical models commonly used for fitting rheogram data. They all rely on the fact that the data were acquired in a linear fashion. See text for definition of variables.

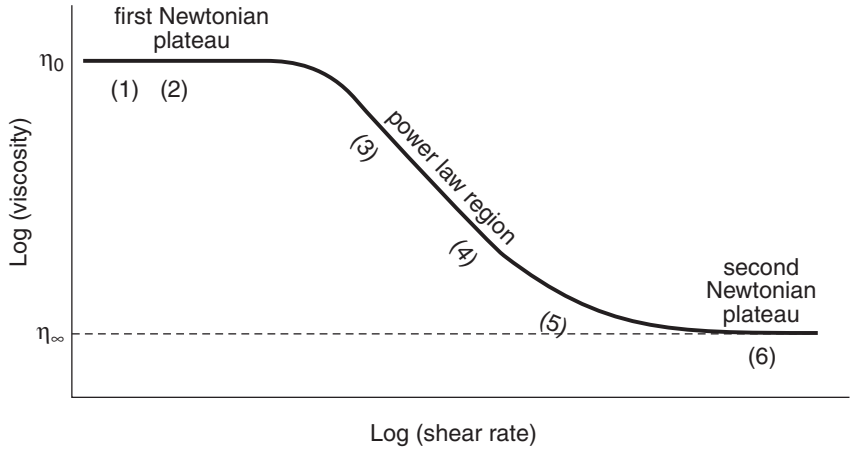

Figure H1.1.4 A complete flow curve for a time-independent non-Newtonian fluid. η_0 and η_∞ are the viscosities associated with the first and second Newtonian plateaus, respectively. Regions (1) and (2) correspond to viscosities relative to low shear rates induced by sedimentation and leveling, respectively. Regions (3) and (4) correspond to viscosities relative to the medium shear rates induced by pouring and pumping, respectively. Regions (5) and (6) correspond to viscosities relative to high shear rates by rubbing and spraying, respectively.

have some positive *y*-axis intercept. Several empirical models are available for fitting the data, but they all rely on the fact that the data were acquired in a linear fashion. If the range of the data is very wide (more than three orders of magnitude), then they will not fit the data well. The linear models are as follows (Figure H.1.1.3):

1. Newtonian model:
   stress = viscosity × shear rate
2. Power Law model:
   stress = consistency coefficient × (shear rate)^n

where the consistency coefficient is equivalent to apparent viscosity and n is the flow behavior index. This model allows for a change in slope (viscosity); if $n < 1$, the material is shear thinning (i.e., pseudoplastic), and if $n > 1$, the material is shear thickening (i.e., dilatant).

3. Bingham model:
   stress = yield stress + (plastic viscosity × shear rate)

This is a straight-line fit shifted up the *y* axis to accommodate a yield stress.

4. Casson model:

$$(\text{stress})^{1/2} = (\text{yield stress})^{1/2} + [(\text{Casson viscosity})^{1/2} \times (\text{shear rate})^{1/2}]$$

where Casson viscosity is the viscosity derived from fitting the Casson model. This slight variation of the Bingham model is often used for dispersions and emulsions.

Carrean-Yasnda Model (full curve)	$\frac{\eta - \eta_\infty}{\eta_0 - \eta_\infty} = \frac{1}{[1 + (k_1\dot{\gamma})^2]^{m_{1/2}}}$
Cross Model (full curve)	$\frac{\eta_0 - \eta}{\eta - \eta_\infty} = (K\dot{\gamma})^m$
For portions of the Cross equation that predict portions of the complete flow curve:	
Power Law (predicts power law region)	$\eta = k\dot{\gamma}^{n-1}$
Williamson (predicts first Newtonian plateau and power law region)	$\eta = \eta_0 - k\dot{\gamma}^{n-1}$
Sisko (predicts power law region and second Newtonian plateau)	$\eta = \eta_\infty + k\dot{\gamma}^{n-1}$

Figure H1.1.5 Empirical models that are used to predict the complete flow curve of non-Newtonian fluids or portions of the complete curve. In the full-curve models, K is a constant with time as its dimension and m is a dimensionless constant. See text for definition of other variables in equations.

5. Herschel-Bulkley model:

$$\text{stress} = \text{yield stress} + [\text{consistency coefficient} \times (\text{shear rate})^n]$$

This is the most versatile of all the above models, allowing for curvature and a *y*-axis shift.

RHEOMETER DATA: THE COMPLETE FLOW CURVE

When a rheometer is used to collect viscosity data, the range covered is typically logarithmic and is therefore much wider than data from a viscometer. The data are generally plotted in a different fashion, as a graph of viscosity versus shear rate on a log-log plot. These curves have a typical shape represented by an initial plateau at low shear rate called the zero-shear viscosity (η_0), a final plateau at high shear rate called the infinite-shear viscosity (η_∞), and a linear portion linking them called the power law region. If all these characteristics are present, then the data can be considered to be a complete flow curve. Often, however, some of the high-shear or low-shear data will be absent. Typically, the controlled-rate rheometer will have problems probing the zero-shear viscosity, while the controlled-stress instrument may not reach the infinite shear viscosity (see section on rheometers; Figure H.1.1.4).

If some or all of this curve is present, the models used to fit the data are more complex and are of two types. The first of these is the Carreau-Yasuda model, in which the viscosity at a given point (η) as well as the zero-shear and infinite-shear viscosities are represented. A Power Law index (m_1) is also present, but is not the same value as n in the linear Power Law model. A second type of model is the Cross model, which has essentially the same parameters, but can be broken down into submodels to fit partial data. If the zero-shear region and the power law region are present, then the Williamson model can be used. If the infinite shear plateau and the power law region are present, then the Sisko model can be used. Sometimes the central power law region is all that is available, and so the Power Law model is applied (Figure H.1.1.5).

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Measuring the Viscosity of Non-Newtonian Fluids

For simple fluids, also known as Newtonian fluids, it is easy to predict the ease with which they will be poured, pumped, or mixed in either an industrial or end-use situation. This is because the shear viscosity or resistance to flow is a constant at any given temperature and pressure. The fluids that fall into this category are few and far between, because they are of necessity simple in structure. Examples are water, oils, and sugar solutions (e.g., honey; *UNIT H1.3*), which have no dispersed phases and no molecular interactions. All other fluids are by definition non-Newtonian, so the viscosity is a variable, not a constant. Non-Newtonian fluids are of great interest as they encompass almost all fluids of industrial value. In the food industry, even natural products such as milk or polysaccharide solutions are non-Newtonian.

Two protocols are presented for non-Newtonian fluids. Basic Protocol 1 is for time-independent non-Newtonian fluids and is a ramped type of test that is suitable for time-independent materials. The test is a nonequilibrium linear procedure, referred to as a ramped or stepped flow test. A nonquantitative value for apparent yield stress is generated with this type of protocol, and any model fitting should be done with linear models (e.g., Newtonian, Herschel-Bulkley; *UNIT H1.1*).

Basic Protocol 2 is for time-dependent non-Newtonian fluids. This type of test is typically only compatible with rheometers that have steady-state conditions built into the control software. This test is known as an equilibrium flow test and may be performed as a function of shear rate or shear stress. If controlled shear stress is used, the zero-shear viscosity may be seen as a clear plateau in the data. If controlled shear rate is used, this zone may not be clearly delineated. Logarithmic plots of viscosity versus shear rate are typically presented, and the Cross or Carreau-Yasuda models are used to fit the data. If a partial flow curve is generated, then subset models such as the Williamson, Sisko, or Power Law models are used (*UNIT H1.1*).

Basic Protocols 1 and 2 both require a significant amount of common methodology that is described in Strategic Planning.

STRATEGIC PLANNING

This section describes common steps designed to measure the viscosity of non-Newtonian materials using rotational rheometers. The rheometer fixture that holds the sample is referred to as a geometry. The geometries of shear are the cone and plate, parallel plate, or concentric cylinders (Figure H1.1.1). The viscosity may be measured as a function of shear stress or shear rate depending upon the type of rheometer used.

If multiple procedures are performed, a qualitative measure of time dependence can be gained. This is achieved by linking two ramps in an up-and-down sequence and examining the area of hysteresis. A second option is to connect three segments as an up ramp, a peak hold step, and a down ramp sequence; this option has the effect of altering the area of hysteresis because the sample is broken down more effectively before the down step.

Before attempting to use either type of flow protocol, the appropriate geometry must be selected and the sample must be loaded into the device. There are certain guidelines that should be followed when selecting a geometry and when setting conditions for sample loading.

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Choosing a Geometry Type

If the sample is a simple (unfilled) liquid (i.e., a transparent or translucent liquid without particles or fibers), a simple cone-and-plate geometry should be selected for testing. Examples of such materials are honey, gum solutions (e.g., xanthan, gellan, carboxy methyl cellulose or CMC), filtered fruit juices, oils, corn syrups, weak gels, and other simple protein or polysaccharide solutions (e.g., albumen).

A filled material, such as ketchup, mustard, unfiltered juices, pastes, emulsions, or dispersions, has particles or droplets dispersed throughout its bulk. The size of such entities needs to be known if a cone-and-plate geometry is to be used, because the gap set in such a geometry is fixed, ranging from ~10 μm for a low-angle cone to ~120 μm for a high-angle cone. The rule in such circumstances is that the gap used must be approximately ten times the size of the particles or droplets.

Aggregates or flocculates must be accounted for when considering the size of such entities. If the above conditions cannot be satisfied, then a parallel-plate or concentric-cylinder system must be selected. Parallel-plate geometries may be set to a user-defined gap ranging from 1000 μm to values determined by the ability of the sample to support its own mass without slumping (normally 2000 to 3000 μm). For concentric-cylinder systems, the gap is defined by the radius ratio of the rotor and the stator.

Solid samples such as chocolate, cheese, or dough naturally lend themselves to testing on a parallel plate because they can be molded or pressed into a sheet. The sheet in turn may be conveniently punched into discs that are the same size as the plates.

Stiff gels that are unfilled are best categorized as solids and should be cut into discs from a cylindrical sample taken by means of a cork borer.

Coarse slurries, such as concentrates of fruit or vegetable pulp and meat or fish pastes with a high water content, are often best tested in a concentric-cylinder system. This is especially true if there is a question about settling or stability. Slip films could be formed at the rotor face if parallel plates are used under these circumstances. (Samples that exhibit syneresis such as yogurt or high-fat-content materials are also cause for concern.)

Choosing a Geometry Size

The choice of geometry size depends upon a coarse viscosity grading of the sample. High-viscosity materials need a small-surface-area geometry, whereas low-viscosity materials require a large-surface-area geometry. Thus, if multiple sizes of geometry are available, the size should be selected based on the ease with which a sample moves in its container. Honey is normally a medium- to high-viscosity material and so is suitable for a medium (~4-cm diameter) or small (~2-cm diameter) cone. A large geometry (~6-cm diameter) should be used to measure apple juice, with its low viscosity. It is important to bear in mind that many materials appear to be immobile but will flow quite readily when disturbed. Materials with this property include tomato ketchup or paste. This type of behavior is associated with an apparent yield stress.

If the sample viscosity is very low (i.e., close to the appearance of water) and a large cone-and-plate or parallel-plate geometry is not available, an alternative is to use a concentric-cylinder arrangement. The size of this system in terms of its surface area should also be at a maximum. Therefore, the use of large-diameter cup-and-bob systems or the so-called double concentric-cylinder system is advisable. A hybrid system called the Mooney-Ewart geometry is also a possible candidate as long as the sample is unfilled. This geometry is a concentric cylinder with a cone and plate as the base. The design has similar advantages to the double concentric cylinder in terms of increased surface area.

Choosing a Geometry Material

Several materials are typically used to manufacture measuring geometries for rheometers. Steel resists solvents and functions under a wide temperature range, but has high inertia. Acrylic or transparent plastic can be sensitive to solvents and has very low inertia, but functions within a limited temperature range. Aluminum, either plain or anodized, resists some solvents, functions within a wide temperature range, and has a low inertia. Composites have similar attributes to acrylic, but are not transparent and function within a wider temperature range. Exotic metals, such as titanium, have similar attributes to steel, but have a lower inertia. In general, the low-inertia materials are the most flexible, because they do equally well for low-viscosity materials and weak gels as for rigid samples. Large steel geometries cause problems in measurement due to their density (inertia); aluminum, composites, and titanium are better choices. Acrylic is an ideal geometry material in many respects, but will not tolerate temperatures >40°C. Its transparency also makes acrylic a good choice for transparent samples.

Other Factors in Selecting a Geometry

If a sample contains volatiles such as solvents, it can change in viscosity as they are lost to the atmosphere. To prevent this, geometries often are supplied with accessories to cover the free sample edge and minimize evaporation. Generally referred to as solvent-trap covers, they should be used if there is a likelihood of drying. Another strategy to eliminate loss of volatiles is to paint a thin film of an inert liquid, such as a silicone oil, on the exposed face or meniscus of the sample. As long as the oil has a low viscosity and the sample is not oil soluble, this should not affect the results. This technique is successfully used with dough samples.

To eliminate sample slippage at the interface, certain styles of geometry will have machined grooves or cross-hatching on their shearing surface. These types of geometries are useful but add uncertainty to the absolute value of the gap. This means that they should be used only if slip is anticipated as a problem.

Setting Up a Geometry

Rheometers will have individual means of attaching a geometry to the drive system or torsion transducer. The manufacturer's instructions should be followed for setup, such that the geometry dimensions are loaded or manually typed into the software for calculating stress and strain (rate) from the raw parameters of torque and displacement (speed). Setting a reference point or bias or mapping the air bearing may also be required. Finally, a value for the geometry inertia must be calibrated or entered. These procedures should be followed so that all the required geometry information is in the software.

Next, the zero-gap reference position (the contact point where the stator and the rotor just touch) should be set using the software routines for bringing rotor and stator into contact. After this, the gap used for the test itself can be set. Generally, the zero-gap referencing procedure should be done at the temperature of interest for testing. Some instruments control temperature using an environmental chamber or oven, whereas others will heat or cool the stator. If the latter is the case, then for temperatures that are well away from ambient, the gap can be referenced at ambient and the temperature of interest set with the sample in place to aid thermal conductivity. This is reasonable if the instrument has a gap temperature compensation factor. Most "autogap" instruments will have this facility built into the firmware that controls the rotor position with respect to the stator.

There are several methods for referencing the zero gap. The deceleration of the rotor as it spins is used by some instruments; others use an electrical circuit between the rotor and

the stator, which indicates the point of contact, or a normal force transducer, which senses the force of contact. The deceleration technique is the most widely available and should be used as a default method unless otherwise indicated in the instrument manual.

Loading the Sample

After setting the gap relative to the zero-gap reference, the sample is loaded into the gap. The separation of the rotor and stator are set to a large value to allow for easy access. For a cone-and-plate or parallel-plate geometry, an aliquot of sample is transferred by pipet or spatula, depending upon its fluidity, to a position centered around the axis of the rotor on the stator. For concentric-cylinder geometries, the sample should be dispensed into the bottom of the cup.

A pipet should be used for dispensing simple liquids. A syringe should not be used because of the high-shear environment in the contracted flow at its tip. Disposable plastic pipets are well suited for this task, as they may be modified to generate a uniform cross-section by removing the tip with scissors. As a general rule, it is better to overestimate the amount of sample needed than to underestimate. Whereas excess sample is relatively easy to remove, an artifact can be generated by repeating the loading process to add more sample. A significant amount of shear is generated in the sample when the gap is closed to its measurement, and thus repeating this process should be avoided.

Many instruments equipped with automatic gap setting will allow the operator to impose a gradual gap closure profile, using a linear speed decrement or even an exponential speed decrement. The latter is especially useful for weak gels (e.g., yogurt) or structured materials suspected of being thixotropic. Instruments with normal force sensors often allow the gap to be closed until a low normal force value is exceeded. This results in a slow stepwise closure for as long as the sample is capable of relaxing to accommodate the narrowing gap. For samples that are gel-like, this technique is less suitable because the sample is unlikely to relax. While using any of these techniques, the sample should be left undisturbed until the gap reaches its expected value.

Sometimes a two-step gap closure is an effective way to achieve reproducible sample loading. In this technique, a trim gap is set whose value is a small increment of the set gap (e.g., gap plus 10%). When this gap is attained, the sample is trimmed around the edge of the geometry using a flat-bladed spatula. It is important to restrict the movement of the rotor while trimming. After trimming the sample, set the final gap, which is specified by the geometry, such that the sample has a slight bulge around the perimeter. This technique is rarely necessary for a concentric-cylinder geometry, because the annulus of the cup is more readily accessible.

The last step, after the sample is loaded in place, is to cover the sample with a solvent-trap cover or silicone oil film if necessary. Once this is done, the first test can be performed.

BASIC PROTOCOL 1

MEASURING VISCOSITY WITH NONEQUILIBRIUM RAMPED OR STEPPED FLOW TESTS

This test is best for time-independent non-Newtonian fluids and can be used to gain a reconnaissance of the sample's performance, for example, where flow begins (apparent yield stress) and whether the sample appears to be time dependent or independent. This protocol is also suitable for determining whether a sample is Newtonian or non-Newtonian. The measured apparent yield-stress value is test dependent, so changing the ranges of shear covered also changes the apparent onset of flow. The hysteresis seen with time-dependent samples is also qualitative and will change with a different test range.

Use these tests carefully! Often it is best to perform these tests as a first approximation of a sample before performing steady-state tests (see Basic Protocol 2).

Materials

Sample, stored in an appropriate clean container (sealed if solvent loss is an issue)
Controlled-rate or controlled-stress rotational rheometer with
Computer control and appropriate software for instrument control, data acquisition, and model fitting
A selection of fixtures with various geometries of different sizes (e.g., diameters, cone angles, and gaps)

Set up and run test

1. Load a sample on a controlled-rate or controlled-stress rotational rheometer and equilibrate to the test temperature (see Strategic Planning).
2. Program the software for a start point of 0 sec^{-1} and an end point of 100 to 500 sec^{-1}.

   If the range of the geometry is exceeded, a software warning will normally occur when these values are entered.

   Controlled-rate operation is also available for most controlled-stress rheometers. The stress range measured with this protocol can subsequently be used to set up exact stresses of interest. If controlled-rate operation is not available, a start point of 0 Pa and an end point of 100 Pa should be programmed. These values will not be suitable for all samples (e.g., an end point of <1 Pa should be used for samples like water, and 100 Pa should be used for samples like ketchup).
3. Set the linear- or log-ramp toggle, if present, to linear. Program the software for the desired test temperature.

   This test is usually carried out at or close to the ambient temperature. If a different temperature is desired, the software should be programmed with an equilibration step prior to the shear step.
4. Set up a time for covering this continuous ramp range, generally 2 to 5 min for simple liquids and 5 to 15 min for complex fluids.

   If a stepped ramp is used, the number of points and a point-time interval should be entered. This is calculated to take the same time as above (e.g., 120 to 300 points at one point per second or 240 to 600 points at two points per second). The exact timing of the test is irrelevant unless the experiment is intended to reproduce work done previously, in which case the previous protocol should be adhered to as closely as possible.
5. *Optional:* If a looped test is desired, add a second ramp that is the reverse of the first. Do not change the conditions if a symmetric hysteresis loop is required.
6. Save the software settings and set up the on-screen plotting capability to give appropriate information.

   Typically, a graph of shear stress versus shear rate is used (linear axes). If multiple y axes are an option, viscosity should be added as a second y axis.
7. Run the test, save the data, and transfer the file to whatever analysis software is to be used.

   Generally the rheometer manufacturer's software is adequate, although files may be exported to spreadsheet programs or calculation packages (e.g., Mathematica, Wolfram Research).
8. Clean the rheometer and geometry according to the manufacturer's instructions.

Analyze data

9. Analyze the data, treating each ramp separately. Apply linear models (e.g., Newtonian, Power Law) based on the appearance of the curve(s).

   A straight line suggests the Newtonian model; a straight line with a positive y intercept suggests the Bingham model. A curve suggests the Power Law model and the presence of a positive y intercept suggests the Herschel-Bulkley model (UNIT H1.1).

10. Note down or print out the values obtained for viscosity or *k* (consistency coefficient), yield stress, and the flow behavior index, and make note of any calculated loop area.

    The calculated loop area is in proportion to the amount of time-dependent behavior and is not to be used for direct quantification. Samples with controlled loading conditions and the same test protocols can be compared.

BASIC PROTOCOL 2

MEASURING VISCOSITY WITH EQUILIBRIUM FLOW TESTS

Equilibrium flow tests are designed to generate as much of the complete flow curve (*UNIT H1.1*) as possible. They are logarithmic stepped ramps with steady-state criteria built into each step. Thus, a step does not conclude until these criteria are met or a certain maximum time is exceeded. These tests are therefore much longer in duration than the ramps in Basic Protocol 1. Equilibrium tests are preferred for time-dependent samples because they run at a rate determined by the sample's own natural response time. At low stresses and shear rates, each point will take minutes to perform. As the sample shear thins, the step times get faster. The response of even a time-dependent sample is converted into a time-independent response.

Materials

Sample, stored in an appropriate clean container (sealed if solvent loss is an issue)
Controlled-rate or controlled-stress rotational rheometer with
  Computer control and appropriate software for instrument control, data acquisition, and model fitting
  A selection of fixtures with various geometries of different sizes (e.g., diameters, cone angles, and gaps)

Set up and run test

1. Load a sample on a controlled-rate or controlled-stress viscometer or rotational rheometer and equilibrate to the test temperature (see Strategic Planning).

2. Program the software for a procedure with a start point of the nearest order of magnitude to the lowest shear rate or stress that can be set for that geometry.

   Typically, this value is ~10^{-3} sec^{-1} for a controlled-rate instrument or ~0.01 Pa for a controlled-stress instrument.

3. Set the linear- or log-ramp toggle, if present, to logarithmic for the ramp and program the software for the desired test temperature.

   This test is usually carried out at or close to the ambient temperature. If a different temperature is desired, the software should be programmed with an equilibration step prior to the shear step.

4. Enter an endpoint of 100 to 1000 sec^{-1} for a controlled-rate instrument or 1000 times the start stress for a controlled-stress instrument.

   The exact start and end points of the ramp can be decided from the ramp performed in Basic Protocol 1. A stress of 1/10th to 1/100th of the apparent yield stress to the nearest order of magnitude should be set.

For controlled-rate devices, the lower limit is less critical. The high-shear end of the ramp can be used to determine the upper limit of stress or shear rate.

Some samples will tend to leave the gap at high rotational speeds.

5. Determine the number of steps to be used.

   Five to ten steps should be used for each order of magnitude of stress or shear rate.

6. Set the steady-state criteria for each step and a step maximum time.

   Typically a running average of the data is taken repeatedly during each step. The averaging time should be on the order of 5 to 30 sec, and at least 50 such segments should be included in each step. Thus a single step could last 250 to 1500 sec if steady-state is not achieved. The most recent average is compared with the previous average and the percent difference is calculated. An acceptable tolerance for this percent difference, somewhere between 5% and 10%, should be set. The final part of the acceptance criterion is the number of times the percent difference is within tolerance before steady state is declared for that point. Three consecutive iterations is a good figure.

   For most samples, a step maximum time of 5 to 25 min should be set, so as to interrupt the step if steady state is unlikely to be achieved. All of the acceptance parameters can be considered as a sliding scale. A fast equilibrium flow test can be not much better than a continuous ramp. If the sample is time dependent with slow rebuild kinetics, then the times should be pushed to their longest limits. Sample stability is an issue, so if the sample is likely to dry or gel at the temperature of interest, the analysis should be carried out quickly (i.e., with shorter step maximum times).

7. Save the software settings and set up the on-screen plotting capability to give appropriate information.

8. Run the test, save the data, and transfer the file to whatever analysis software is to be used.

   Generally the rheometer manufacturer's software is adequate, although file may be exported to spreadsheet programs or calculation packages (e.g., Mathematica, Wolfram Research).

9. Clean the rheometer and geometry according to the manufacturer's instructions.

Analyze data

10. Plot the data as viscosity versus shear rate on logarithmic axes.

11. Choose a model to analyze the data, using the one that gives the best (lowest) standard error.

    The models used are typically either the Cross model or the Carreau-Yasuda model (UNIT H1.1), if a complete curve is generated. A complete curve has both plateaus present (zero and infinite shear; see Figure H1.1.4).

12. To assess the yield behavior of a sample, plot the data as viscosity versus stress.

    If a sample shows a very sharp drop in viscosity over a narrow stress range, then it can be considered to have an apparent yield stress.

    Comparison of materials is often more successful on a plot of viscosity versus stress.

13. If the data at low shear are noisy, examine the data table.

    If the first data points do not reach equilibrium but exceed the maximum time allowed, then the test may be too demanding for the instrument as it is set up. The run should be repeated with a more sensitive geometry (larger surface area) or a more sensitive transducer (controlled-strain instruments only), which may improve the data. Prolonging each step may or may not improve data.

COMMENTARY

Background Information

For non-Newtonian fluids that are time independent, the use of linear ramps and the models mentioned in Basic Protocol 1 can be useful in obtaining a reasonably broad picture of a material's performance, and this method certainly is useful in predicting how well a liquid might pour or pump. These tests become inadequate if the sample is strongly time dependent. The faster the ramp is performed, the greater the error will be. The problem with time-dependent behavior is that the rate of structure breakdown is a function of the change in shear (test controlled), whereas the rate of structure buildup is a function of the kinetics of structure assembly (sample controlled) and the change in shear (test controlled). Thus, the faster the test, the greater the likely discrepancy between the breakdown of structure and its rebuilding.

A logarithmic ramp addresses some of these problems by increasing the data density in the critical zone where flow begins. A typical logarithmic ramp spans from 0.01 to 100 Pa or 0.01 to 100 sec^{-1}, or so as to give round numbers that are orders of magnitude apart. The same time frames for continuous and stepped ramps apply here, but the effect is to magnify the low-shear end of the curve. This can be programmed in the software by switching the linear- or log-ramp toggle (see Basic Protocol 1, step 3). A yield stress measured here will still be in error, but it will probably be seen as a smaller value. Thixotropic loop test hysteresis will also be somewhat reduced but will still be imperfect and qualitative only.

If a logarithmic ramp is performed, then the data should not be fit with linear models (*UNIT H1.1*). These data should be plotted as viscosity versus shear rate on logarithmic axes and the Carreau-Yasuda or Cross models (or subsets) should be used instead. It is unlikely that the zero-shear plateau will be seen in these types of tests. For a complete flow curve, the equilibrium tests described in Basic Protocol 2 should be used.

The linear ramps (continuous or stepped) have the advantage of speed and work best for simple shear-thinning or shear-thickening materials. They should compare with single-point determinations from other equipment if the sample is Newtonian. If the sample is non-Newtonian then there may be discrepancies. This will occur if the single-point device uses a complex shear field (e.g., Zahn or Ford cup). Data from a rotational rheometer using cone-and-plate or concentric cylinders should agree within limits of ~2% to 5% for Newtonian samples and ~10% for non-Newtonian time-independent samples. Time-dependent samples can be orders of magnitude apart.

The equilibrium flow test in Basic Protocol 2 is very useful for measurement of time-dependent samples. The nonequilibrium tests in Basic Protocol 1 are much quicker, but can give results that are hard to reproduce if the sample is time dependent. The equilibrium flow test is far longer but may only need to be performed once.

The complete flow curve allows for the response of the test sample to be predicted at rest (zero-shear behavior), when being poured out of a container (early Power Law region), when being pumped (middle Power Law region), or when being processed or consumed. Each part of the curve is important in assessing a sample's suitability.

There are additional benefits to the measurement of the complete flow curve with a controlled-stress rheometer. The low stresses give data at extremely low shear rates, allowing for good characterization of the zero-shear viscosity (η_0). This parameter is proportional to the molecular weight of polymers in solution or as melts. As the M_n or M_w (number and weight average molecular weight, respectively) increases, so does the value of η_0. The radius of curvature of the plot of viscosity versus shear stress or shear rate as it leaves the zero-shear plateau and begins to shear thin is related to the molecular weight distribution of the polymer in the sample. Monodisperse samples will give a small radius of curvature, whereas polydisperse samples will give a large radius of curvature. The same principles are true for particle size and size distribution or droplet size and size distribution in dispersions and emulsions.

Critical Parameters and Troubleshooting

The rheometer and the geometry should be in good condition (i.e., clean and free from visible damage). Results can be affected by pitting and dings. When attached to the instrument, the geometry should be concentric to the axis of rotation and should hang vertically so as to rotate without eccentric oscillation. Temperature control devices should be calibrated with a thermocouple and corrected for any offset. Prior to running a sample that is not a

standard, the instrument should be calibrated with a traceable standard oil. The measured viscosity of the standard at a given temperature should be within 2% to 5% of the certified value.

The best way to detect problems occurring during the test is to visually inspect the sample rather than to focus on the data appearing on the monitor. One typical problem resulting from time-dependent behavior is irregular changes in rotor speed as the sample breaks down. Switching from a nonequilibrium to an equilibrium test can improve this problem, especially if a controlled-stress device is used.

The same type of behavior can be seen if slip is occurring, but usually it is apparent that only part of the sample is being sheared, rather than the entire bulk. Some workers mark the sample with a notch or a pen to observe the change during shear. To test for slip, a series of experiments using a parallel plate geometry and varying gaps should be run. If the data are not consistent, then slip is occurring. At that point it is necessary to use a serrated geometry or to modify the smooth surface. Slippage can be reduced by gluing fine-grade sandpaper to the surface of the parallel plates.

If the sample is highly filled or elastic it may edge fracture and be expelled at high shear rates. This is usually very easy to spot, and there is little to be done about it in a rotational instrument. Highly elastic samples are measured in a capillary device at high shear rates where edge fracture is not a problem.

Anticipated Results

The common method for graphic display of data generated using Basic Protocol 1 is a graph of shear stress on the *y* axis versus shear rate on the *x* axis. Most fluid foods will be shear thinning and their resulting graph will appear similar to a pseudoplastic or pseudoplastic plastic curve as shown in Figure H1.1.2. In some cases two curves are recorded for each sample. The first is with an increasing shear rate or stress (up curve). After the completion of this step the shear rate or stress is then decreased by the same rate to the initial value (down curve). A comparison of the up and down curves will usually match one of the following cases. In the first case, both curves will be superimposed. This suggests that the fluid is time independent. In the second case, the up curve will be above the down curve. This appears as a loop on the shear stress versus shear rate graph. This loop is called a thixotropic loop and the area inside the loop is dependent on the degree of time dependency of the material. This area is also dependent on the rate of increase of the shear rate (up curve) and the rate of decrease of the shear rate (down curve). Because the loop area is not solely dependent on the material, it is not often measured. Its appearance is simply taken as an indication that the fluid has time-dependent flow properties. In this case the fluid is often measured with the method described in Basic Protocol 2.

The common method for graphic display of data generated using Basic Protocol 2 is a log-log plot of viscosity versus shear rate (Figure H1.1.4). However, the covered shear-rate range is often not wide enough to characterize the complete curve as shown in Figure H1.1.4. When using a controlled-stress rheometer, the first Newtonian plateau and power region are measured. When controlled-rate instruments are used, only the power region is usually measured. The second Newtonian plateau region is usually not obtainable with rotational instruments. Instruments such as a high-pressure capillary rheometer are needed for these high–shear rate measurements.

Time Considerations

Analysis of a sample using Basic Protocol 1 will take 15 to 30 min. Using the equilibrium test in Basic Protocol 2 will take 25 to 60 min per sample, with longer times required as the number of data points increases.

Key References

Pettitt, D.J., Wayne, J.E.B., Renner Nantz, J.J., and Shoemaker, C.F. 1995. Rheological properties of solutions and emulsions stabilized with xanthan gum and propylene glycol alginate. *J. Food Sci.* 60:528-531, 550.

This work reports rheological measurements of food polymer suspensions and emulsions. These are typical of a large number of foods and their rheological behaviors.

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Viscosity Determination of Pure Liquids, Solutions and Serums Using Capillary Viscometry

This unit describes a method for measuring the viscosity (η) of Newtonian fluids. For a Newtonian fluid, viscosity is a constant at a given temperature and pressure, as defined in *UNIT H1.1*; common liquids under ordinary circumstances behave in this way. Examples include pure fluids and solutions. Liquids which have suspended matter of sufficient size and concentration may deviate from Newtonian behavior. Examples of liquids exhibiting non-Newtonian behavior (*UNIT H1.1*) include polymer suspensions, emulsions, and fruit juices. Glass capillary viscometers are useful for the measurement of fluids, with the appropriate choice of capillary dimensions, for Newtonian fluids of viscosity up to 10 Pascals (Newtons m/sec $^{-2}$) or 100 Poise (dynes cm/sec $^{-2}$). Traditionally, these viscometers have been used in the oil industry. However, they have been adapted for use in the food industry and are commonly used for molecular weight prediction of food polymers in very dilute solutions (Daubert and Foegeding, 1998). There are three common types of capillary viscometers including Ubelohde, Ostwald, and Cannon-Fenske. These viscometers are often referred to as U-tube viscometers because they resemble the letter U (see Fig. H1.3.1).

Capillary viscometers are ideal for measuring the viscosity of Newtonian fluids. However, they are unsuitable for non-Newtonian fluids since variations in hydrostatic pressure during sample efflux results in variations in shear rate and thus viscosity. This unit contains protocols for measuring the viscosity of pure liquids and solutions (see Basic Protocol) and serums from fruit juices and pastes (see Alternate Protocol).

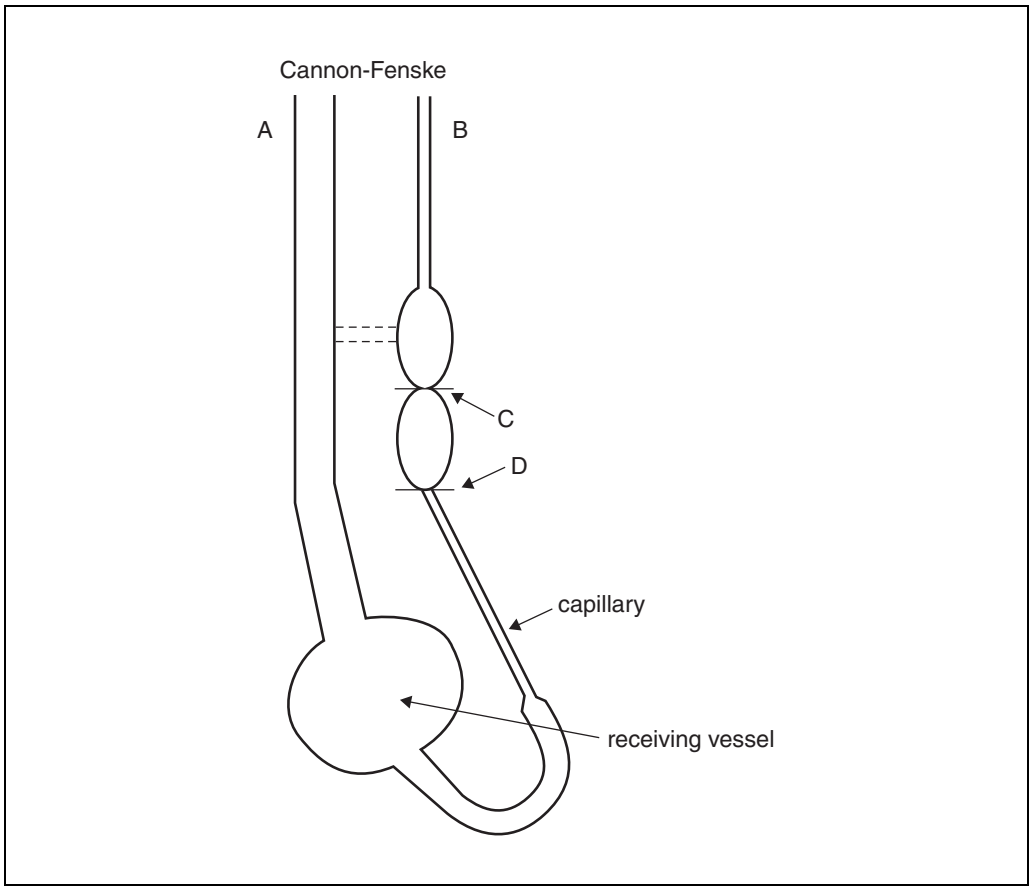

Figure H1.3.1 Schematic of a glass capillary (U-tube) viscometer.

Contributed by Jody Renner-Nantz
Current Protocols in Food Analytical Chemistry (2001) H1.3.1-H1.3.5

BASIC PROTOCOL

USING CAPILLARY VISCOMETRY TO DETERMINE THE VISCOSITY OF PURE LIQUIDS AND SOLUTIONS

This protocol describes a method for measuring the viscosity of pure liquids and solutions by capillary viscometry. The sample is loaded into a Cannon-Fenske viscometer. The time required for the sample to flow between two time points on the viscometer is used to calculate the kinematic viscosity or viscosity.

Materials

Cleaning solvent appropriate for sample
Pure liquid or solution sample

Temperature-controlled 30°C water bath consisting of:
- 4.5-gallon glass tank
- Temperature-controlled immersion water circulator
- Apparatus to hold viscometer vertically in water bath

Thermometer
Capillary viscometer tube (Cannon-Fenske)
Solvent wash bottle
10-ml syringe fitted with a syringe filter holder (25-mm diameter)
Nylon membrane filters (25-mm diameter and 0.8-µm pore size)
Tubing with ID that fits over tube B of viscometer (see Figure H1.3.1)
Rubber suction bulb that connects to tubing
Faucet-adapter type aspirator
Stopwatch (±0.2 sec)

Load and equilibrate viscometer

1. Turn on the temperature-controlled water bath and set the temperature to 30°C. Allow the water bath to heat and equilibrate for 30 min before measuring the viscosity of any fluids. Verify the water bath temperature with a thermometer before proceeding.
2. Clean the Cannon-Fenske viscometer by placing the appropriate solvent into a solvent wash bottle. Continuously flush and aspirate the solvent through the viscometer until it is clean.
3. Pass dry filtered air through the viscometer to remove final traces of solvent. Mount viscometer in water bath.
4. To remove lint, dust, or other solid material from the sample, filter sample using a 10-ml syringe fitted with a syringe filter holder and a nylon filter.
5. To charge the sample into the viscometer discharge the sample from the syringe with filter directly into tube A of the viscometer. Discharge enough sample to fill the receiving vessel (see Figure H1.3.1) to about ¾ full.

   The same sample volume should be used for all subsequent measurements.
6. Make sure that the viscometer is in a vertical position in the bath.
7. Equilibrate the sample in the water bath for ~10 min.

Make the measurement

8. With the suction bulb connected to one end of the tubing, connect the other end of tubing to tube B and apply suction to draw the sample through the capillary and to a level above the etched line noted as C.
9. Remove the tubing and measure the efflux time using a stopwatch by allowing the sample to flow freely down past mark C, then measure the time it takes for the meniscus to pass from mark C to mark D in seconds.

Table H1.3.1 ASTM[a] Sizes, Viscosity Ranges, and Constants for Cannon-Fenske Type Viscometer Tubes

ASTM size	Viscosity range (cSt)[b]	Approx. constant
25	0.5 to 2	0.002
50	0.8 to 4	0.004
75	1.6 to 8	0.008
100	3 to 15	0.015
150	7 to 35	0.035

[a]American Society for Testing and Materials.
[b]A centistoke is a unit of kinematic viscosity equal to the kinematic viscosity of a fluid having a dynamic viscosity of 1 centipoise and a density of 1 g/cm^3.

Calculate the viscosity

10. Calculate the kinematic viscosity (η') of the sample in centistokes (cSt with units of cm^2/sec) by multiplying the efflux time in seconds by the viscometer constant (Table H1.3.1). Repeat measurements as necessary.

    $\eta' = At$

    where η' is the kinematic viscosity, A is the viscometer constant (cSt/sec), and t is the efflux time in seconds.

    If the viscometer constant A is unkown, it may be determined by measuring the efflux time of a fluid with a known kinematic viscosity and calculating the viscometer constant A with the equation above.

11. The viscosity (mPa or cP) can be determined with the kinematic viscosity and density of the fluid, ρ (g/cm^3), with the following equation.

    $\eta = A\eta' = A\rho t$

USING CAPILLARY VISCOMETRY TO DETERMINE THE VISCOSITY OF SERUMS, FRUIT JUICES, OR PASTES

ALTERNATE PROTOCOL

This method is an adaptation of the Basic Protocol for measuring the viscosity of pure liquids and solutions. The °brix (*UNIT H1.4*) of the sample is adjusted to a desired value by dilution. In many protocols, a nominal value of 5 °brix is the accepted target value for dilution. The sample is then filtered to remove particles that would plug the capillary tube of the viscometer, and the serum viscosity is measured in a Cannon-Fenske viscometer.

Additional Materials *(also see Basic Protocol)*

Refractometer with automatic temperature compensation feature
Stomacher lab blender with sample bags (Fisher)
Laboratory centrifuge
Side-arm Erlenmeyer flask
Buchner funnel
Whatman no. 1 filter paper

1. Turn on refractometer.
2. Determine the initial °brix of the fruit juice, serum, or paste.
3. Calculate the amount of water needed to adjust the °brix to 5.0 using the following equation where C_I is the °brix of the original sample, C_F is the desired °brix (5.0

°brix), W_I is the weight of the original sample, and W_F is the final weight of the diluted sample:

$W_I = C_F W_F / C_I$

4. Add water to dilute the sample to 5.0 °brix.
5. Blend the sample in the stomacher for 2 min or until the °brix measurement of the sample is consistently 5.0 ± 0.1 °brix.
6. Centrifuge the sample for 15 min at 10,000 × *g*, 25°C. Decant the supernatant into a beaker, measure the °brix, and record.
7. If there are remaining any large particles, vacuum filter the sample using a side-arm Erlenmeyer flask fitted with a Buchner funnel and Whatman no. 1 filter paper.
8. Filter the sample three times using the syringe filtering apparatus described in the Basic Protocol.
9. Repeat steps 4 through 9 of the Basic Protocol.

COMMENTARY

Background Information

The principle of operation of capillary viscometers is described by the Poiseuille equation where the rate of liquid flow (V/t) through the viscometer can be determined by the following (Steffe, 1996):

$V/t = (\rho' gl + \Delta\rho)\Pi R^4/8\eta l$

where ρ' is the density of the fluid, g is the force due to gravity, l is the length of the capillary tube, and $\Delta\rho$ is the pressure difference between the entrance and exit of the capillary tube. The equation can be rearranged and further simplified to account for all the constants that characterize the viscometer. This also assumes that the difference in height of the two liquid columns is relatively constant during the time required for flow. Thus, the only pressure difference across the liquid is due to the weight of the liquid. With these conditions:

$\eta = A\rho t$

where A is a constant that incorporates all the parameters that characterize a viscometer.

A simple way to evaluate the viscosity of an unknown (η_2) is to compare its viscosity to that of a known (η_1) such that:

$\eta_2 = \{\rho_2 t_2/\rho_1 t_2\}\eta_1$

where ρ_1 and ρ_2 are the densities of the known and unknown, respectively.

The viscosity of a fluid corrected for its density is also known as the absolute or kinematic viscosity.

Critical Parameters and Troubleshooting

There is a range of capillary tubes available for use depending on the viscosity range of the samples to be measured. Typical Cannon-Fenske capillary tube sizes and viscosity ranges are listed in Table H1.3.1. The viscometers are available calibrated or uncalibrated. Uncalibrated viscometers require the user to calibrate them using fluids with know viscosities.

Since viscosity is temperature dependent, the temperature-controlled water bath should be checked daily for accuracy.

Table H1.3.2 Viscosities of Sucrose Solutions Measured by a Capillary Viscometer[a]

Sucrose (%)	Viscosity × 10^2 (dynes s/cm^2)	Standard deviation
5	1.086	0.012
10	1.217	0.011
20	1.542	0.011
30	2.107	0.008

[a]Values from Young and Shoemaker (1991).

The volume of sample used for each measurement should be kept constant.

Anticipated Results

Viscosity will vary depending on the sample. Typical results for viscosity of sucrose solutions are shown in Table H1.3.2.

Time Considerations

Once the constant-temperature water bath has reached 30°C, measuring the viscosities of pure solutions like standard oils requires ~15 min per sample. The most time-consuming part of the measurement is cleaning and drying the viscometer between samples. Preparing fruit pastes and juices for viscosity determination will take ~1 hr for two to four different samples and then an additional ~15 min/sample for the viscosity measurements.

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Key Reference

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Detailed discussion about using capillary viscometry.

Contributed by Jody Renner-Nantz
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Measuring Consistency of Juices and Pastes

BASIC PROTOCOL

An empirical method for measuring the consistency of various coarse suspensions of juices, preserves, jams, pastes, and other highly viscous products is described in this protocol. In previous units, protocols were given for the measurement of viscosity of fluid foods (*UNITS H1.2 & H1.3*). These are fundamental measurements in that the actual viscosity is measured. Viscosity is a property of the fluid and its value should not depend on the method of measurement. In this unit a popular empirical test is described. The Bostwick consistometer is used to determine sample consistency by measuring the distance in centimeters that a material flows under its own weight during a given time interval, which in this case is 30 sec at 25°C. Other temperatures can be used as long as all measurements are made at the same temperature. Viscosity and consistency are very temperature dependent (Eley, 1995; Weaver, 1996).

Consistency will also be influenced by the natural variation of soluble solids in fruit juices. Because the soluble solids level changes during processing, consistencies are evaluated among juices at a fixed level of soluble solids. The soluble solids content is determined by refractometry, which measures the percent of sugar (sucrose) by weight at the temperature indicated on the instrument. The scale used is the Brix scale, which is equivalent to percent sucrose concentration. A common Brix level used by the tomato industry to evaluate tomato paste consistency is 12 °Brix. Soluble solids (measured in degrees Brix) are often used as an indication of sugar content and maturity level in fruits, vegetables, and their industrial products (Marsh et al., 1990).

Materials

Fruit juice or paste
Brix refractometer
Stomacher laboratory blender with sample bags (e.g., Thomas Scientific)
Bostwick consistometer (Fisher Scientific)

1. Apply a drop of a fruit juice or paste sample to a Brix refractometer, hold the refractometer perpendicular to a light source, and determine the initial Brix (percent soluble solids) value for the sample.

   Adding bubbles to the sample, which will give incorrect readings, should be avoided.

2. Calculate the amount of water needed to adjust the °Brix value to 12 using the following equation:

   $$W_i = (C_f \times W_f)/C_i$$

   where W_i is the initial weight of the sample, W_f is the final weight of the diluted sample, C_f is the final desired Brix value, and C_i is the initial Brix value of the sample.

3. Blend the sample and added water in a stomacher laboratory blender fitted with a sample bag until the °Brix value of the sample is consistently 12 ± 0.1.

   A minimum of 250 g sample should be prepared for each reading.

   Samples with lower Brix values can also be measured as long as their concentrations are consistent from experiment to experiment.

   The stomacher is used to evenly blend the water into the paste without incorporating air into the sample. Other blenders of this type could also be used.

4. Adjust sample temperature to 25°C.

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5. Close the gate of a Bostwick consistometer and adjust leveling screws until the leveling bubble indicates that the consistometer is level.
6. Fill the consistometer reservoir to the point of overflow (e.g., ~300 g of 12 °Brix paste) and remove excess from top of reservoir using a spatula.
7. Release the gate and let the sample flow for 30 sec. At the end of this period, measure the distance the paste flowed from the gate to the leading edge of the sample in centimeters and record this as the Bostwick value.
8. Rinse consistometer with water and dry.
9. Make at least three replicated readings with fresh samples.

COMMENTARY

Background Information

In the food industry it has often been difficult to obtain true viscosity measurements (*UNITH1.1*) of complex fluid foods such as coarse fruit suspensions. These are usually non-Newtonian suspensions. Fruit concentrates are dispersions of solid particles (pulp) in aqueous media (serum). Their rheological properties are of interest in practical applications related to processing, storage stability, and sensory properties. Expensive rheometers are often not available in quality control and product development laboratories. However, viscosity is nonetheless an important quality factor of these products.

In order to obtain an estimate of viscosity, many empirical tests have been developed in the food industry. Each test characterizes the viscosity of a product by some type of empirical measurement, which is largely influenced by product viscosity. The value produced by such measurements may be influenced by other rheological properties of the food as well as the instrument used for the measurement. As long as the same instrument is used in all measurements of the food products the measured values may be ranked in order of their viscosity. Generally such empirical measurements of fluid foods are said to yield consistency values. In the food industry, E. P. Bostwick of the U.S. Department of Agriculture, Canned Fruits and Vegetable Service developed a widely used empirical measurement of consistency using the Bostwick consistometer. In theory, as the Brix value of a sample increases, the Bostwick consistency value should decrease. However, if during food processing additives such as corn starches or syrups are included in the food formulation, the correlation between the Brix concentration and Bostwick consistency values could be lost. Here the term consistency may be considered as related to viscosity, although the exact numerical conversion may not be known.

Critical Parameters and Troubleshooting

Because consistency is strongly affected by temperature and concentration of soluble solids, both parameters should be carefully controlled during sample preparation and measurement. Filling the Bostwick reservoir, leveling the product, and releasing the gate must be done in a timely manner to prevent separation of serum and changes in temperature.

Anticipated Results

The consistency of a sample will vary depending on a number of factors including the ripening stage of the initial product and processing conditions. Some typical °Brix values and their standard derivations are: tomato juice, 5.5 ± 0.2; tomato puree, 7.3 ± 0.1; ketchup, 12.0 ± 0.01; and tomato paste, 12.0 ± 0.1. Bostwick values for the same products are: 8.5 ± 0.2, 9.4 ± 0.09, 22.0 ± 0.08, and 6.5 ± 0.02, respectively. Both Brix and Bostwick values can vary among similar products depending on their individual processing conditions and formulations. For both product consideration and consumer appeal, products with a high consistency or viscosity (i.e., with low Bostwick values for the same Brix concentration) are very valuable.

Time Considerations

The largest time factor in this procedure is associated with adjusting the Brix level of the samples and equilibrating their temperatures. The time required for sample preparation and measurement is estimated to be 15 min per sample.

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Key References

McCarthy, K.L. and Seymour, J.D. 1994. Gravity current analysis of the Bostwick consistometer for power law foods. *J. Texture Stud. 25:207-220.*

Compared experimental measurements for Newtonian and power law fluids to theoretical predictions and showed that the apparent viscosity predicted by the Bostwick measurement must be correlated with flow behavior during processing and thus could be very useful to incorporate into food process design and control.

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General Compressive Measurements

BASIC PROTOCOL

The compressive measurement of a food material is important to understanding issues related to materials handling (e.g., bruising) as well as some textural properties. This unit describes mechanical testing of food specimens and the necessary sample preparation required to obtain specimen geometries generally subjected to compressive tests. In this protocol, a mechanical testing machine equipped with parallel plates is used to achieve uniaxial compression at a desired loading rate. This measurement provides an avenue to determine force and deformation up to the point of rupture (yield), and from these data determine the stiffness of the material under investigation. From a stress/strain curve, further details can be gathered on the state of the specimen at failure, as well as the apparent elastic modulus or Young's modulus of elasticity. Young's modulus is the ratio of the extensional (tensile) stress divided by the corresponding extensional strain of an elastic material, measured in uniaxial extension. It is represented by the symbol E in units of pascals (Pa). All of this information may be derived by physical analysis of the curves or by using applicable computer software.

The mechanical testing machine applies a load to a sample by compressing the food material between two parallel plates. The rigidity of the material dictates which type of load cell should be used in making measurements; however, samples can range from rigid solids to soft biological materials such as gels or apple parenchyma tissue. This protocol will focus on strongly structured food tissue systems and weakly structured gel materials. For a discussion of sample geometry selection, see Commentary.

Aspect ratios of certain types of prepared specimens vary from author to author. In addition, no standardized deformation rates have been established for many of the food specimens subjected to compressive measurements. Thus, comparison of results between different laboratories is difficult if test conditions vary. As a result, valid comparisons of data may only be possible internally.

NOTE: Safety protocols from the manufacturer of the specific mechanical testing machine should be followed closely. For example, limit switches should be adjusted to prevent any overtravel, up or down, by the cross-head.

Materials

Material specimen (prepared or intact)
Razor blades, cork borer, wire cutting device
Mechanical testing machine linked to either a chart recorder or computer (with data recording and analysis software)
Parallel plates, stainless steel or Teflon
Lubricating oil (recommended)

Prepare sample

NOTE: Preparation of specimens requires that dimensions be measured precisely. It is assumed that the dimensions of the compressed specimen are small with respect to the dimensions of the contacting surfaces (plates) to ensure that the sample remains in contact with the plates. Occasionally, it is desirable to compress a sample in its natural state (i.e., no specimen cutting).

1a. *For cubic or rectangular solid samples:* Cut flat surfaces using a razor blade or a parallel-wire cutter. For agricultural specimens (e.g., fruits and vegetables), prepare samples with an aspect ratio (height to diameter) of ~1 and a sample height of one to several centimeters. Be sure that samples have smooth parallel sides.

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1b. *For cylindrical solid samples:* Use a cork borer or similar cutting tool of appropriate diameter. If necessary, apply a lubricant (e.g., mineral oil) to the cork borer to facilitate removal of the cut specimen. Cut samples to the desired length using a razor blade or wire cutting device. For agricultural specimens (e.g., fruits and vegetables), prepare a sample with an aspect ratio (height to diameter) of ~1 and a sample height of one to several centimeters. Be sure that the ends of the cylindrical specimen are smooth and parallel.

Readily available cork borers range in diameter from 7.1 to 22.2 mm.

1c. *For gel samples:* Pour gelling solution into a mold with known dimensions and an aspect ratio (height to diameter) of ~1. Allow gel to form by cooling or heating, as appropriate.

2. Allow the specimen to equilibrate to the temperature at which it is most likely stored or used (e.g., refrigerated, ambient, or elevated temperature).

This assists in mimicking the conditions most commonly encountered by the specimen.

Set up testing device

3. Choose a load cell that has the capability to manage the expected maximum load exerted on the specimen.

4. Turn on the mechanical testing machine and attach parallel plates.

5. Turn on the chart recorder or computer.

If using computer software, procedures specific to the software will need to be followed.

6. For test equipment driven by computer software, enter required information—e.g., specimen dimensions, cross-sectional area, desired calculations, and the rate at which the sample will be deformed (cross-head speed or deformation rate).

Table H2.1.1 Nomenclature and Corresponding Units

Symbol	Parameter	S.I. unit
A_0	Initial cross-sectional area	m^2
D	Mean product diameter	m
d (ΔL)	Deformation (change in length)	m
E	Young's modulus of elasticity	Pa
F	Force, load	N
L (h)	Length (height) of compressed specimen	m
L_0 (h_0)	Original length (height) of specimen	m
t	time	s
u_z	Velocity in z direction (deformation rate)	m s^{-1}
ΔX	Change in width of specimen	m
X_0	Original width of specimen	m
$\dot{\varepsilon}_B$	Biaxial extensional strain rate	s^{-1}
ε_{eng}	Engineering strain	dimensionless
ε_h	Hencky strain	dimensionless
ε_f	Maximum strain at failure	dimensionless
η_B	Biaxial extensional viscosity	Pa·s
σ_B	Biaxial stress	Pa
σ_{eng}	Engineering stress	Pa
σ_h	True (corrected) stress	Pa
σ_f	Maximum stress at failure	Pa
μ	Poisson's ratio	dimensionless

Measure compression

7. Apply lubricating oil to specimen surfaces that contact the plates.

   This will minimize friction between the contact surfaces.

8. Place the specimen on the lower flat plate and move the upper plate to a position near the top surface of the specimen. Measure the distance between the plates to obtain the sample height (*h*).

   This may be required, depending on the computer software.

9. Zero the position of the upper plate and zero the load on the load cell.

10. Select a desired deformation rate and select a point at which to stop the test.

    Generally, testing will cease at any point beyond rupture (maximum stress) or at a chosen % strain.

11. Start the test.

    CAUTION: *The cross-head will begin to move down. Keep hands clear!*

12. Record the force (*F*) versus deformation (*d*) data for the test.

    See Table H2.1.1 for variables and units used in these calculations.

13. At any point during the test, calculate engineering stress (σ_{eng}) and engineering strain (ε_{eng}) from the force/deformation data set or plot:

$$\sigma_{eng} = \frac{F}{A_0} \qquad \varepsilon_{eng} = \frac{d}{L_0}$$

    where F is the applied force, A_0 is the initial cross-sectional area of the specimen, and L_0 is the initial length of the specimen (i.e., the sample height, h, in step 8).

Analyze data

14. For large deformations (e.g., $d > 25\%$; Peleg, 1985), convert engineering stress and strain to true stress (σ_h) and Henky strain (ε_h) as follows:

$$\sigma_h = \sigma_{eng}(1 - \varepsilon_{eng}) \qquad \varepsilon_h = \ln(1 + \varepsilon_{eng})$$

15. Determine sample strength by measuring the stress at failure (σ_f). To do this, determine the force at failure via a force/deformation plot (see Figure H2.1.1) and convert force to either engineering stress or true stress. Also use deformation at failure (*d*) to calculate the strain at failure (ε_f).

$$\sigma_f = \frac{F}{A_0} \qquad \varepsilon_f = \frac{d}{L_0}$$

16. Determine Young's modulus of elasticity (*E*), which is the slope of the linear portion of the stress versus strain curve. Calculate the stiffness of the specimen from the force/deformation curve.

$$E = \frac{\sigma_h}{\varepsilon_h} \qquad \text{stiffness} = \frac{F}{d}$$

    To correct deformation for an initial curved region of the force/deformation curve (toe region) due to specimen irregularities at the contact surfaces of a specimen with a Hookean

region (where stress is proportional to strain and a linear relationship between stress and strain can be shown), the curve may be continued through the zero stress (force) axis to provide a corrected zero strain (deformation). For specimens with no Hookean region, the zero strain point is determined by a tangent line through the maximum slope at the inflection point of the toe region.

It should be noted that the Young's modulus for convex bodies is calculated differently. It is sometimes desirable to determine the mechanical properties of a specimen (e.g., an apple, potato, grape) in its natural state. This can provide valuable information for those interested in materials handling. These materials have convex surfaces in contact with flat loading plates; thus, the assumptions made with cube and cylindrical geometries no longer hold. Using the Hertz model (Mohsenin, 1970), Young's modulus of elasticity can be calculated for these convex bodies provided Poisson's ratio is known or estimated. In this case, Young's modulus is calculated as follows:

$$E = \sqrt{\frac{16(1-\mu^2)^2 F^2}{14.285 \times Dd^2}}$$

where μ is Poisson's ratio, F is the applied force, d is the deformation, and D is the mean product diameter.

17. Where no linear region is discernable from a force/deformation or a stress/strain curve, use a secant modulus. Construct a secant line from a desired stress (force) point and extend it to the zero strain (deformation) point. Use the slope of this secant line to give the secant modulus.

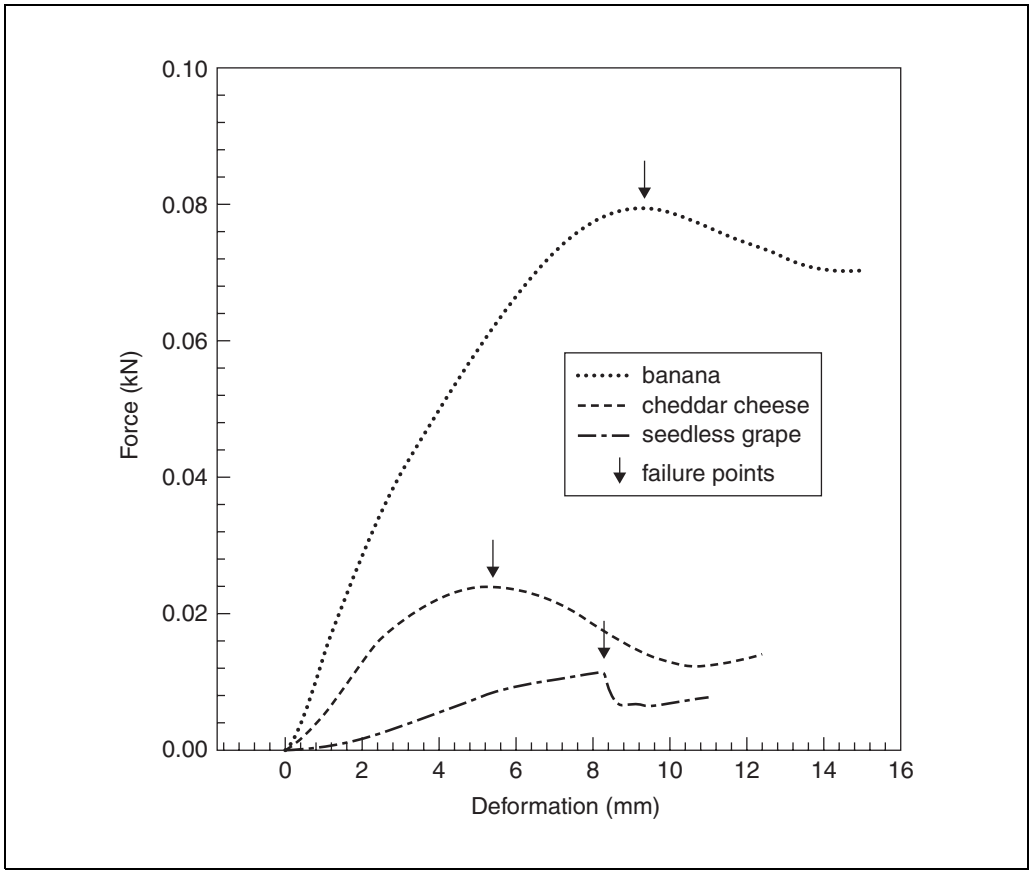

Figure H2.1.1 A force/deformation curve illustrating specimen fracture of a banana (2.5 cm length, 3.0 cm diameter, 5 mm/min deformation rate), cheddar cheese (2.0 × 2.0 × 2.0 cm, 10 mm/min deformation rate), and a seedless grape (2.2 cm length, 1.7 cm diameter, 2 mm/min deformation rate) under uniaxial compression at room temperature.

18. Analyze lubricated squeezing flow to determine biaxial extensional viscosity (η_B), which is calculated from biaxial stress (σ_B) and biaxial extensional strain rate ($\dot{\varepsilon}_B$).

$$\sigma_B = \frac{F}{A} = \frac{Fh}{A_0 h_0} \qquad \dot{\varepsilon}_B = \frac{u_z}{2(h_0 - u_z t)} \qquad \eta_B = \frac{\sigma_B}{\dot{\varepsilon}_B}$$

σ_B and $\dot{\varepsilon}_B$ are determined from force-deformation curves for materials which exhibit squeezing flow behavior (e.g., peanut butter, processed cheese).

COMMENTARY

Background Information

The study of the mechanical properties of a food is important for determining its strength, texture, and deformation characteristics. The geometry, size, and shape of a sample should conform to standards such as those set by the American Society for Testing and Materials (ASTM), or should meet assumptions for use in mechanical tests for formula development (Mohsenin, 1970).

Studies of food rheology require an understanding of stresses, strains, and rates of strain. A normal stress is one that is perpendicular to the surface it is acting on and is either compressive or tensile (Peleg, 1987). A normal stress can be expressed as an engineering stress (magnitude of applied force/initial cross-sectional area of specimen) or a true stress (magnitude of applied force/cross-sectional area of deformed specimen) (Peleg, 1987). Additionally, there are shear stresses, which are parallel to the surface it acts on. Shear stress has the same dimensions as normal stress, but the area is parallel to its direction (Peleg, 1987).

Engineering (apparent) strain is defined as the ratio between the deformation of the specimen and the initial length of the specimen. Deformation is described as the absolute elongation or length decrease in the direction of the applied force (Peleg, 1987). Engineering strain can be considered to be true strain when the deformation is small; however, when the deformation is large, this is no longer true. In this case, the most appropriate definition of true strain would be that defined by Hencky (Peleg, 1987).

The failure characteristics of a food or food material can be measured using compression, tension, or torsion. Of all the available deformation tests, possibly the most common is uniaxial compression (Lelievre et al., 1992). Bulk compression is another type of compression test, but it is seldom used due to the difficulty in applying force by means of hydraulic pressure (Bourne, 1982). The experimental data obtained from the compression of a food material (Figure H2.1.1) is often acquired using instruments such as a mechanical testing machine (Bagley et al., 1988).

A specimen can fail in compression, tension, or shear. A specimen that fractures at 45% to the longitudinal axis has failed in shear (Lelievre et al., 1992). Tensile failure results if the specimen fractures at 90% to the longitudinal axis, and compressive failure is represented by fracture along the longitudinal axis (Lelievre et al., 1992).

Uniaxial compression of a cylindrical shape is a method of measuring the behavior of a Hookean solid. Hooke's law is represented by the following relationship:

$$\sigma_{eng} = E\varepsilon_{eng}$$

where E is Young's modulus of elasticity, σ is stress, and ε is strain. Hooke's law is usually valid for solids under small strains (e.g., <0.25; Steffe, 1996). Under large deformations, Hencky strain (ε_h) should be used. This assumes that the sample maintains a cylindrical shape during compression (Steffe, 1996).

Critical Parameters

There are three principal geometries used for samples subjected to compressive measurements. The two most common are cubic and cylindrical shapes. Cubic samples are commonly used when examining cheese specimens (Ak and Gunasekaran, 1992), while cylindrical samples are common in the compression of polymer gel samples (Tang et al., 1996) and apples (Rebouillat and Peleg, 1988). The third geometry is a convex body such as a sphere. For potatoes and apples, which approximate a spherical shape, it may be possible to compress the intact specimen rather than a cut specimen (Morrow and Mohsenin, 1966). This may be preferred because of the difficulty of correlating compression data of cut specimens with that of whole specimens.

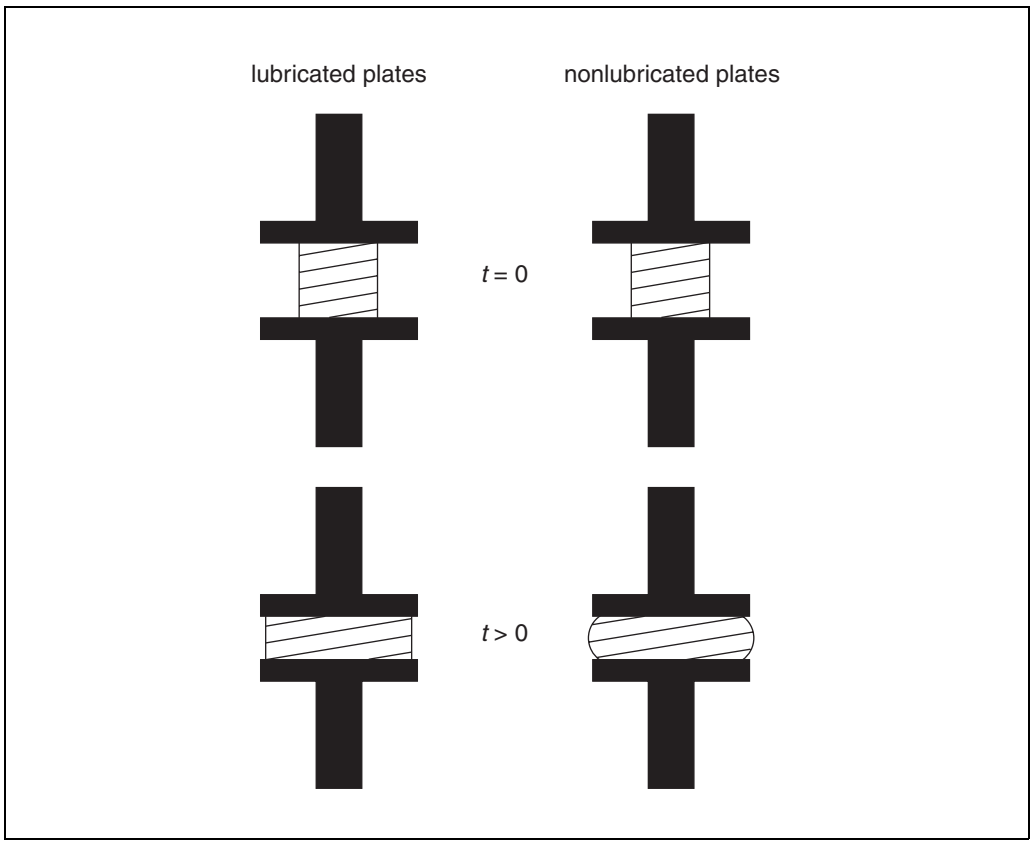

Figure H2.1.2 Specimen compression between parallel plates that are lubricated and nonlubricated.

Certain assumptions are often presented when considering the compression of a specimen. These include homogeneity and isotropy of the sample, the use of contacting plates that are infinitely large, and the use of a specimen whose radius of curvature is large compared to the length of the contacting area (Morrow and Mohsenin, 1966). For normal sizes and shapes of convex agricultural products, these assumptions are generally valid (Morrow and Mohsensin, 1966).

When performing a compression test, lubricated contact surfaces between the specimen and loading plates are generally preferred to nonlubricated surfaces, as lubrication prevents barreling (Figure H2.1.2). Barreling results from friction between the specimen and the contacting surfaces.

Compression of a weakly structured food between parallel plates may achieve squeezing flow (Steffe, 1996). When lubricated parallel plates are used, the result is a form of biaxial extension. Biaxial extension may be used to measure biaxial viscosity, which is a reflection of resistance to radial stretching flow in a plane. Lubricated squeezing flow of a semi-solid specimen (Figure H2.1.3) supports the assumption that specimen deformation is homogeneous and thus extensional viscosity can be evaluated (Rao, 1992).

Ideally, the stress/strain relationship should be independent of the dimensions of a specimen in uniaxial compression (Peleg, 1987). Often, samples that have a height-to-diameter ratio (aspect ratio) ≤1 do not follow this rule. This occurs either from frictional forces or because the food material resists having the fluid squeezed from the matrix. Friction at the contact surfaces can increase the apparent strength of the sample and change its deformation behavior (Peleg, 1987). In fluid-filled samples, resistance to pressure dissipation depends on the length and nature of the path and on the density and microstructure of the sample (Peleg, 1987). Therefore, the greater the diameter of the sample (greater fluid path), the greater the apparent firmness and strength of the specimen.

In uniaxial compression, one dimension of a cylindrical sample is compressed while the area in contact with parallel plates increases (Steffe, 1996). The ratio between the increase

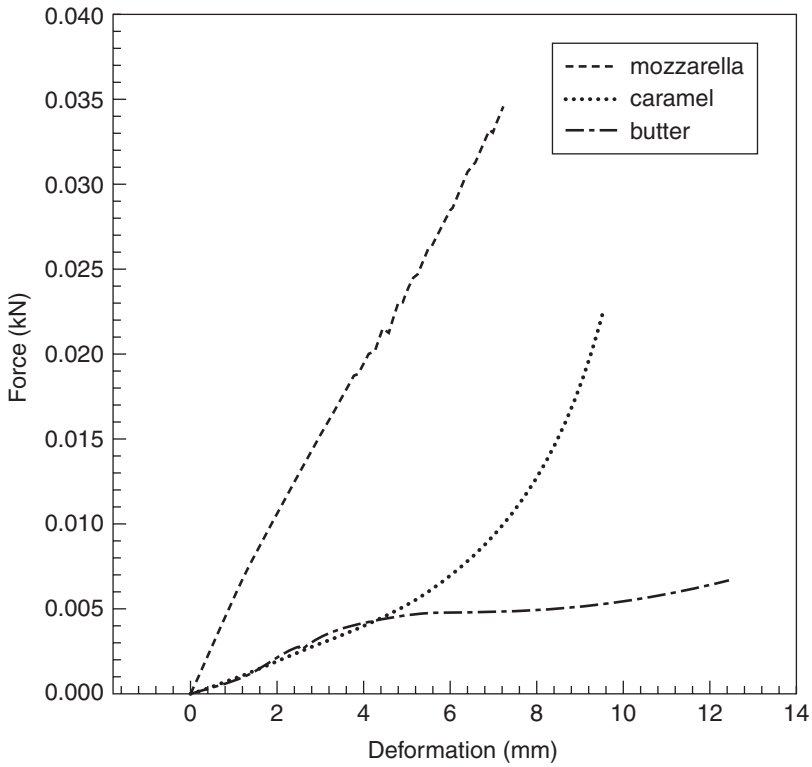

Figure H2.1.3 A force/deformation curve illustrating the lubricated squeezing flow of mozzarella cheese (2.3 cm length, 1.8 cm width, 1.8 cm height, 10 mm/min deformation rate), butter (2.1 cm diameter, 2.4 cm height, 5 mm/min deformation rate), and caramel (2.2 cm diameter, 1.9 cm height, 2 mm/min deformation rate) under uniaxial compression at room temperature.

in diameter (transverse strain) and the decrease in height (axial strain) is called Poisson's ratio (μ):

$$\mu = \frac{\Delta X / X_0}{d / L_0}$$

Compressive measurements provide a means to determine specimen stiffness, Young's modulus of elasticity, strength at failure, stress at yield, and strain at yield. These measurements can be performed on samples such as soy milk gels (Kampf and Nussinovitch, 1997) and apples (Lurie and Nussinovitch, 1996). In the case of convex bodies, where Poisson's ratio is known, the Hertz model should be applied to the data in order to determine Young's modulus of elasticity (Mohsenin, 1970). It should also be noted that for biological materials, Young's modulus or the apparent elastic modulus is dependent on the rate at which a specimen is deformed.

When evaluating force/deformation (Figure H2.1.1) or stress/strain curves, an artifact caused by the seating of the specimen, or a takeup of slack is sometimes observed. This "toe" compensation must be corrected to obtain a zero strain or zero deformation point.

Anticipated Results

Rigid specimens (e.g., apple, cheddar cheese) often exhibit a sudden decrease in force (stress) after a certain amount of deformation (maximum strain). At this point the specimen has fractured. Maximum stress and strain values may vary depending on the chosen specimen. Specimens that are weakly structured and tend to flow under lubricated compression (e.g., mozzarella cheese, marshmallow) demonstrate squeezing flow. As a result, the force (stress) continually increases as the specimen deformation (strain) increases. These materials do not fracture, but continue to stretch radially while under compression. Both rigid and soft specimens of the same material may exhibit varying characteristics depending on the deformation rate and the aspect ratio of each specimen.

Time Considerations

Sample preparation may require as little as 5 min (e.g., cutting a sample cube of cheese) or an hour or more (e.g., preparation of a food polymer solution and pouring in a mold). The time to run a test will depend on the deformation rate and the degree of deformation chosen for the specimen of interest. Typically, uniaxial compression of a specimen can be performed in 5 to 20 min.

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Key References

Bourne, 1982. See above.

A good discussion of the information that can be obtained from uniaxial compression data.

Steffe, 1996. See above.

A helpful analysis of lubricated squeezing flow with problem solving examples and results for selected materials.

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Textural Measurements with Special Fixtures

This unit describes the measurement of textural properties of food using special testing fixtures. For the purposes of the unit, the focus will be on puncture probes, cone penetrometers, shear cells, wire cutting devices, and back extrusion fixtures, all of which lend themselves to textural testing in compression. Using a punch such as a Magness-Taylor puncture probe or a cone penetrometer (Basic Protocol 1) allows one to measure the firmness of a food material. The cone penetrometer, in particular, provides a means for determining a yield value. Shear cells such as those from Warner-Bratzler and Kramer (Basic Protocol 2) allow for different characteristics to be analyzed, as these fixtures combine resistance to cutting with shear, tension, and compression forces on the sample. For some structured foods, such as cheese, a wire cutting device is used to measure hardness. In addition, back extrusion cells (Basic Protocol 3) are useful for determining the force of extrusion in a form of compression-extrusion test. An understanding of human sensory interpretation of texture is essential for selecting appropriate fixtures for textural measurements.

TEXTURAL MEASUREMENTS USING A PUNCTURE PROBE OR A CONE PENETROMETER

BASIC PROTOCOL 1

Puncture probes and cone penetrometers provide a simple means for determining the textural properties of many structured foods (see sample results in Figure H2.2.1).

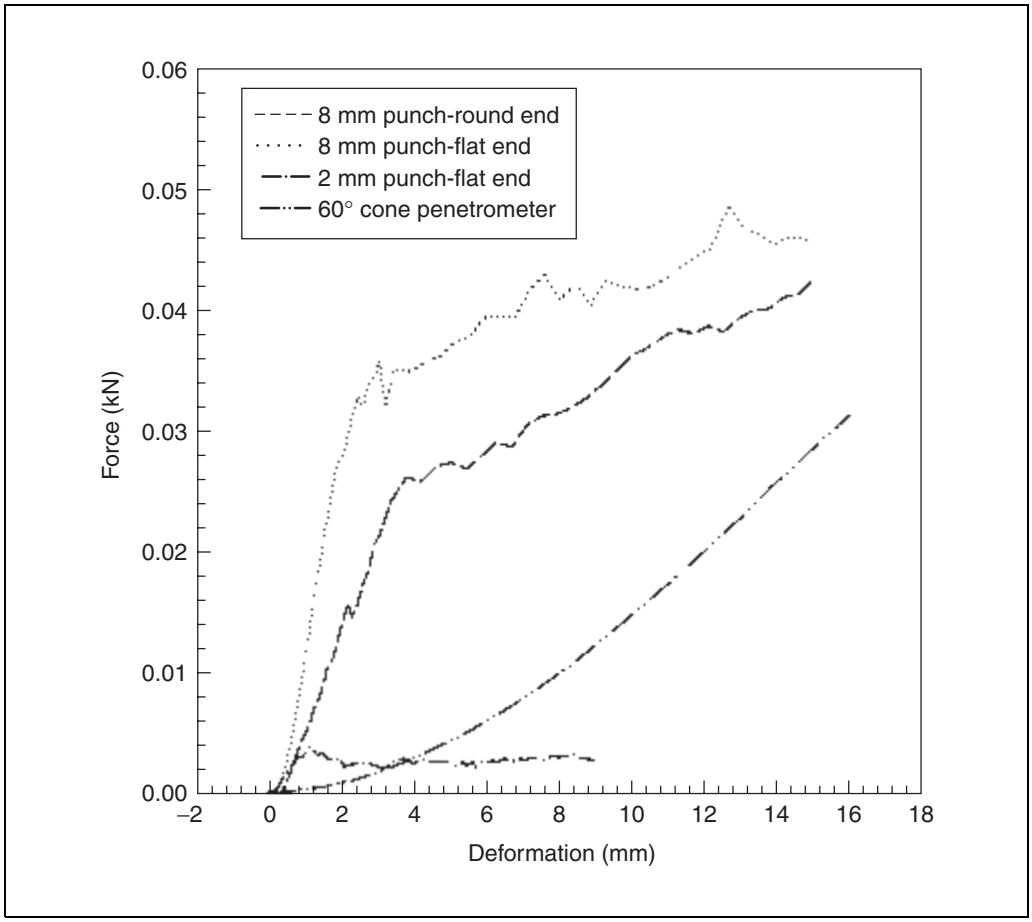

Figure H2.2.1 Force/deformation curves illustrating three puncture probe tests (50 mm/min deformation rate) of an apple specimen and a cone penetrometer test (10 mm/min deformation rate) of cheddar cheese, all at room temperature.

Contributed by Marvin A. Tung and Michael D.H. Rogers

Current Protocols in Food Analytical Chemistry (2001) H2.2.1-H2.2.12

Puncture probes are commonly used for fruits and vegetables, and allow for the determination of force at rupture of the cellular structure. The procedure outlined below is adapted from the method of Bourne (1979). Cone penetrometers are commonly employed for determining firmness and yield value for foods such as margarine and butter, which may be a reflection of the product's spreadability. Quite often it is desirable to use a testing system that provides a constant deformation rate. Additionally, a mechanical testing machine allows for production of a force/deformation curve to further analyze the data.

Materials

Material specimen (prepared or intact)
Mechanical testing machine linked to either a chart recorder or a computer with data recording and analysis software (optional; puncture probes are often hand held)
Testing fixture (select as appropriate):
Puncture probe
Cylindrical probe (either a hand-held instrument or a fixture for a mechanical testing machine), flat or rounded end (Magness-Taylor)
Cone Penetrometer (cone angle and geometry may vary)

Prepare sample

1. Prepare specimen so that the diameter and gauge length of the material are sufficient to meet the requirements for depth of penetration.

   The sample size should be large when compared to the size of the particular fixture in use so to ensure that the entire surface of the specimen remains in contact with the fixture.

   Puncture probe diameter should be held constant during a given series of tests.

2. Allow the temperature of the specimen to equilibrate to the temperature at which it is most likely stored or used (e.g., refrigerated, ambient, or elevated temperature).

   This assists in mimicking the application conditions most commonly encountered by the specimen.

Perform measurements

For hand-held puncture probes:

3a. With one hand, hold the structured food against a rigid surface (e.g., a laboratory bench).

4a. Hold puncture probe perpendicular to the surface to be tested.

5a. With the puncture probe in the other hand and the side of this hand resting on your hip, lean into the puncture probe to provide a uniform rate of force application.

   Try to avoid uneven movements during testing.

6a. For food products such as freshly harvested apples and ripe fruit, continue penetration until probe depth reaches the inscribed line on the probe.

   Often raw vegetables will require that penetration continue until contact with the instrument's splash guard.

   In the case of specimens such as apples, it may be appropriate to remove the epicarp (skin) in the region being tested.

7a. Record the maximum force from the instrument.

For mechanical testing machines:

3b. Choose a load cell that has the capability to manage the expected maximum load to be exerted on the specimen.

4b. Turn on the mechanical testing machine and attach the appropriate fixture (puncture probe or cone penetrometer).

5b. Turn on the chart recorder or computer.

If using computer software, procedures specific to the software will need to be followed.

6b. For test equipment driven by computer software, enter required information—e.g., specimen dimensions, cross-sectional area, desired calculations, and the rate at which the sample will be deformed (cross-head speed or deformation rate).

7b. Place the specimen on the lower flat base plate and move the fixture, attached to the moving cross-head, to a position near the top surface of the specimen. Measure the distance between the lower plate and the fixture to obtain the sample height, which may be required by the computer software and in further calculations.

8b. Zero the position of the fixture in relation to the sample surface and the zero the load on the load cell.

9b. Select a desired deformation rate (dh/dt) and select a distance at which to stop the test.

Testing should cease at the appropriate depth of penetration or at rupture.

10b. Start the test.

CAUTION: *The cross-head will begin to move down. Keep hands clear!*

11b. Record the force/deformation data from the test.

12b. For puncture probes, determine sample strength by measuring the force or stress at rupture. For flat-ended probes, calculate the force engineering stress from the area of the contact surface of the probe (A) and the force at rupture (F) from a force/deformation plot.

$$\sigma_f = \frac{F}{A}$$

It is important to note that the ultimate strength of a specimen may not be the point of rupture. The rupture point may occur prior to the point illustrating the ultimate strength of a material (see Figure H2.2.2).

See Table H2.2.1 for variables and units used in these calculations.

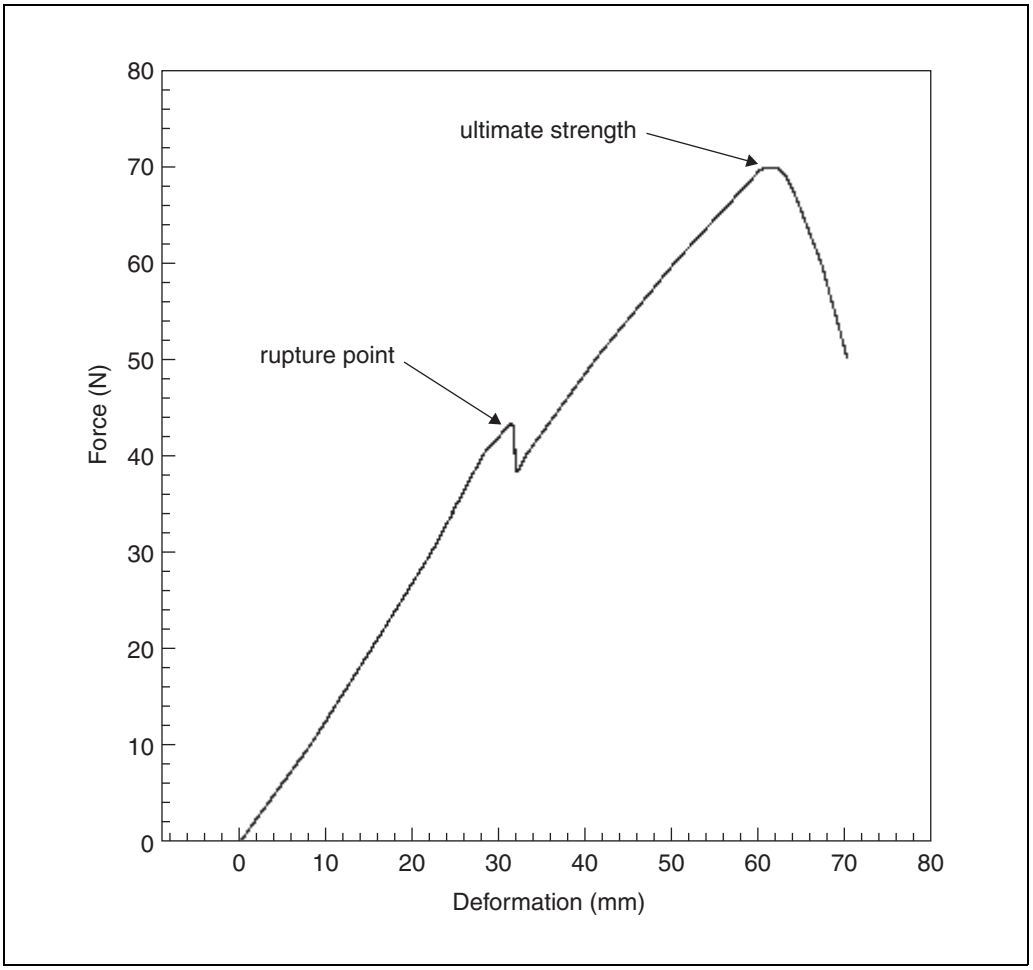

Figure H2.2.2 A force/deformation curve illustrating the potential difference between the rupture point and the ultimate strength of a food specimen (adapted from Mohsenin, 1970).

Table H2.2.1 Nomenclature and Corresponding Units

Symbol	Parameter	S.I. unit
A	Area	m^2
A'	Actual area where F' is applied	m^2
c (K)	Constant	Dimensionless
F	Force	load
F'	Actual force applied for sample	N
f	Firmness of butter	Pa
h	Height of cone	cm
K_c	Compression coefficient	Dimensionless
K_s	Shear coefficient	Dimensionless
l	Length of cone	cm
p	Perimeter	m
r	Radius of cone	cm
dh/dt	Speed of cone penetrometer	cm/s
η_{app}	Apparent viscosity	Pa·s
σ_f	Maximum stress at failure	Pa
θ	Cone angle	Degrees

13b. Calculate the yield value (f) for materials such as margarine and butter, as determined by cone penetrometry, using the following equations from Tanaka et al. (1971).

$$F' = \frac{F}{\cos(\theta/2)}$$

$$A' = \frac{\pi r l}{\cos(\theta/2)}$$

$$r = h\tan(\theta/2)$$

$$l = \frac{h}{\cos(\theta/2)}$$

$$F'/A' = \eta_{app}(dh/dt) + f = \frac{F\cot(\theta/2)\cos(\theta/2)}{\pi h^2}$$

If F'/A' (penetration stress) is plotted versus dh/dt (penetration speed), the slope is the apparent viscosity (η_{app}) and the intercept (penetration stress at $dh/dt = 0$) is the yield value (f).

TEXTURAL MEASUREMENTS USING WARNER-BRATZLER, KRAMER, OR WIRE CUTTING FIXTURES

BASIC PROTOCOL 2

Warner-Bratzler, Kramer, and wire cutting fixtures provide another method of measuring textural components of some food materials. The Warner-Bratzler fixture is useful for measuring the shear stress associated with cutting of meat products. The Kramer shear fixture (Figure H2.2.3) has important applications for textural measurements of products such as peas and peaches. In this case, the force at rupture is a result of both cutting and compression forces. Finally, wire cutting devices (sectilometers; Figure H2.2.4) are important for cheese and butter products. The firmness of the food material is measured as the maximum force encounter on the wire during cutting.

Materials

Material specimen (prepared or intact)
Mechanical testing machine linked to either a chart recorder or computer (with data recording and analysis software)
Testing fixture (select as appropriate; see Background Information):
Warner-Bratzler test cell
Kramer shear/compression cell (single-blade or multiblade cells)
Wire cutting device (sectilometer; single- or multiwire)

Prepare specimen

1. Prepare speciman as appropriate for the testing device to be used:
   a. *For Warner-Bratzler measurements:* Cut specimen so that it is cylindrical in shape.
   b. *For Kramer shear cell measurements:* Cut into small cubes or chunks (i.e., 1 cm lengths) if excessively large.
   c. *For sectilometer measurements:* Cut so that specimen has a width less than the length of the wire(s) on the instrument.
2. Allow the temperature of the specimen to equilibrate to the temperature at which it is most likely stored or used (e.g., refrigerated, ambient, or elevated temperature).

   This assists in mimicking the application conditions most commonly encountered by the specimen.

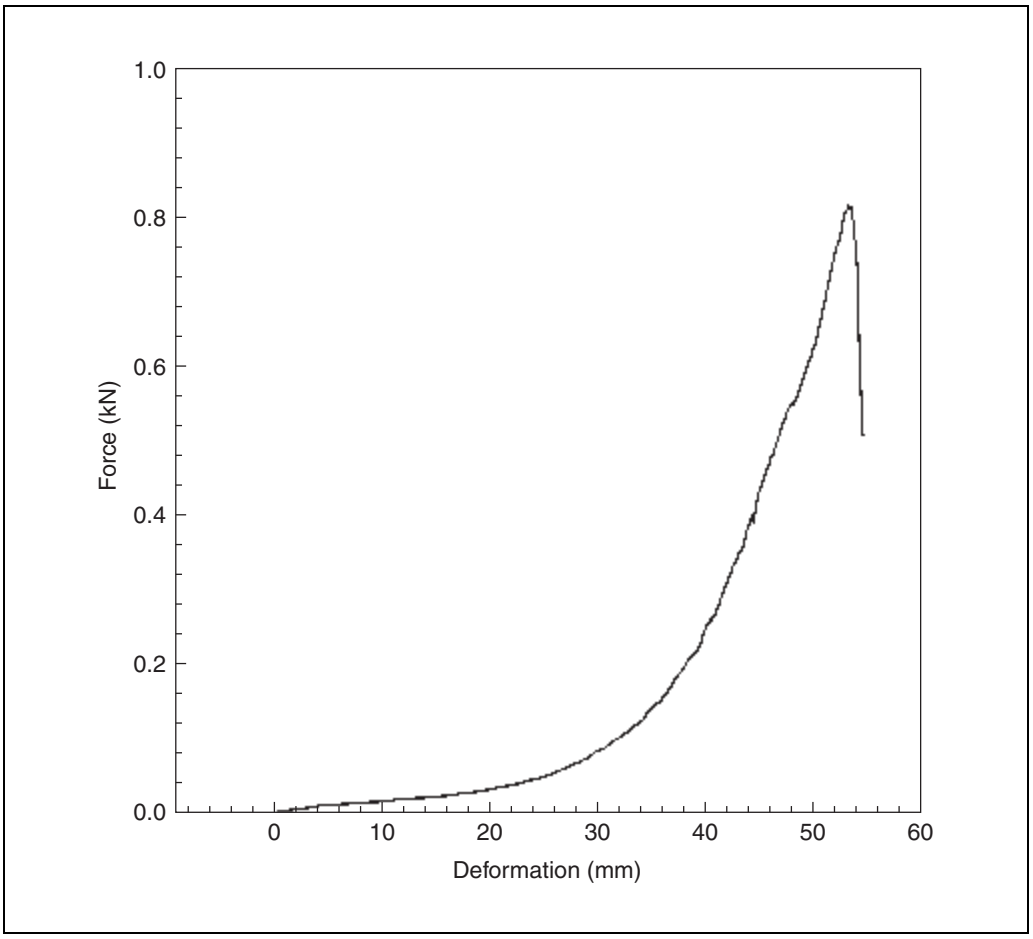

Figure H2.2.3 A force/deformation curve illustrating a compression-extrusion test (10 mm/min deformation rate) for canned green peas using a Kramer shear cell (multiblade) at room temperature.

Perform measurements

3. Choose a load cell that has the capability to manage the expected maximum load to be exerted on the specimen.
4. Turn on the mechanical testing machine and attach the appropriate fixture (Warner-Bratzler, Kramer, wire cutting fixture).
5. Turn on the chart recorder or computer.

   If using computer software, procedures specific to the software will need to be followed.

6. For test equipment driven by computer software, enter required information such as specimen dimensions, cross-sectional area, desired calculations, and the rate at which the sample will be deformed (cross-head speed or deformation rate).
7. Place the specimen on the two metal parts of the lower portion of the Warner-Bratzler fixture or the Kramer shear cell and fill the lower fixture to about half capacity. In the case of a wire cutting device, place the specimen on the lower plate.
8. Move the top fixture to a position near the top surface of the specimen. Measure the distance between the lower plate and the fixture to obtain the sample height, which may be required by computer software or in future calculations.

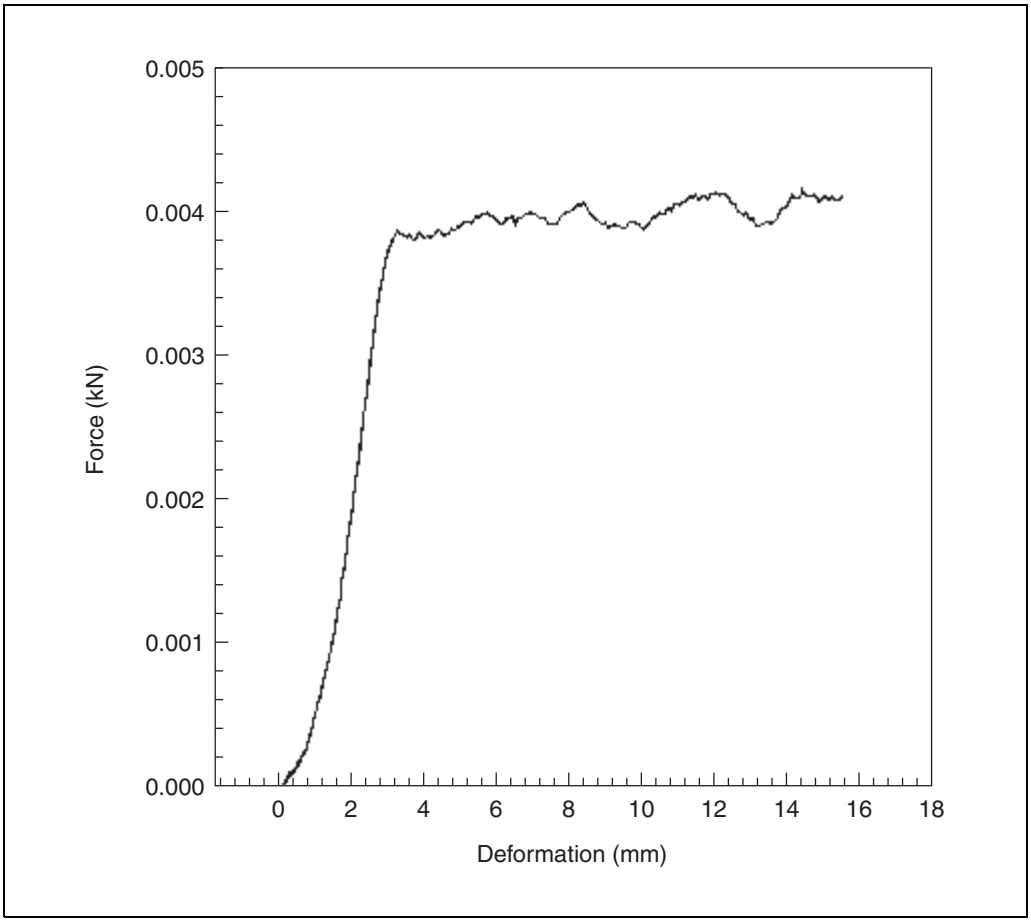

Figure H2.2.4 A force/determination curve illustrating a wire cutting test (10 mm/min deformation rate) for cheddar cheese using a single-wire sectilometer at room temperature.

9. Zero the position of the fixture in relation to the sample surface and zero the load on the load cell.
10. Select a deformation rate of 22.86 cm/min (9 inches/min) for the Warner-Bratzler test. For the Kramer shear cell test or the wire cutting test, select a desired deformation rate.

    Testing should cease at the appropriate depth of penetration or at rupture.

11. Start the test.

    CAUTION: *The cross-head will begin to move down. Keep hands clear!*

12. Record the force/deformation data for the test.

    Measurements made using a Warner-Bratzler fixture, a Kramer shear/compression cell, or a wire cutting device may demonstrate rupture prior to the reaching the ultimate strength of the specimen (Figure H2.2.2). The force at rupture is the most important factor required for textural analysis using these fixtures. A food material being tested using a Kramer cell may fail in either compression, tension, shear, or some combination of the three. Measurements obtained through sectilometer tests allow for the determination of the firmness of the sample. Firmness is the maximum force needed to push the wire through the food.

TEXTURAL MEASUREMENTS USING BACK EXTRUSION

Back extrusion is an important method for determining the yield force required for food materials that are homogeneous and that flow. The yield force is the point where flow is initiated. Using a plunger and a cylinder open at one end (Figure H2.2.5), it is possible to extrude food material back through the annular gap. Extrusion occurs beyond the rupture point of the food. This method is commonly performed with cooked materials such as peas and rice.

Materials

Material specimen (prepared or intact)
Mechanical testing machine linked to either a chart recorder or computer (with data recording and analysis software)
Back extrusion cell (plunger and a container with an open top having a certain annular gap)

Prepare sample

1. Prepare a food specimen that can flow (e.g., fruits, vegetables, liquids, and gels) and is homogeneous in size.
2. Allow the temperature of the specimen to equilibrate to the temperature at which it is most likely stored or used (e.g., refrigerated, ambient, or elevated temperature).

   This assists in mimicking the conditions most commonly encountered by the specimen.

Perform measurements

3. Choose a load cell that has the capability to manage the expected maximum load exerted on the specimen.

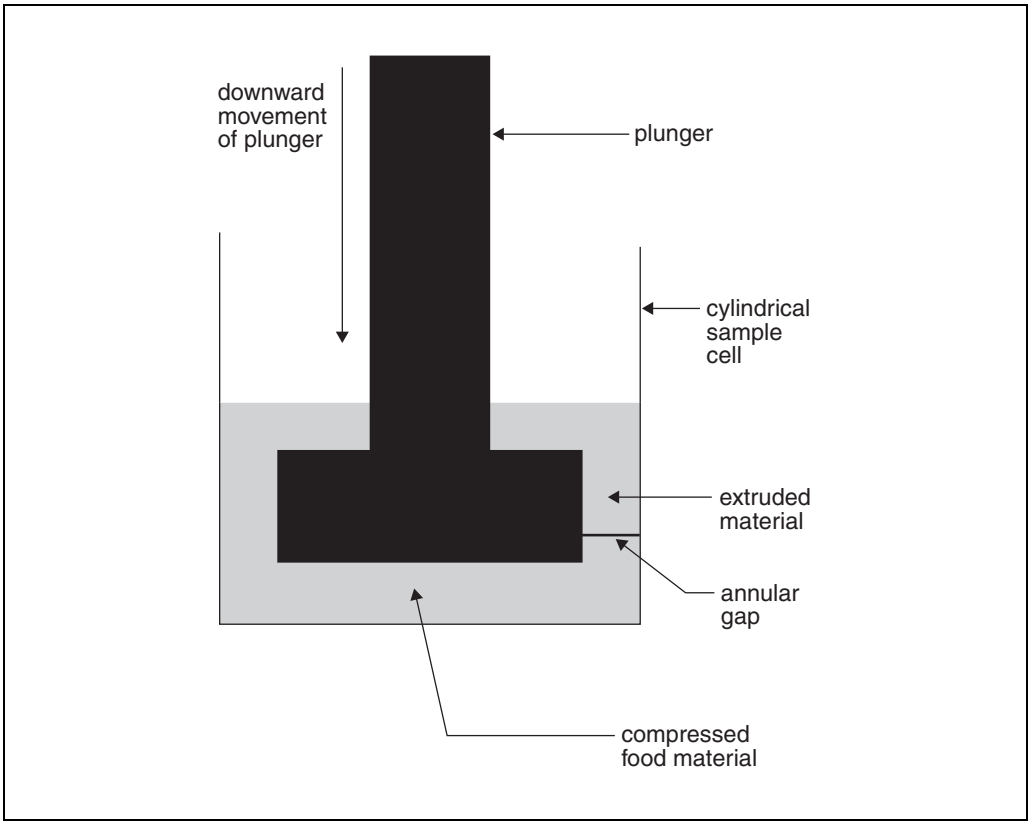

Figure H2.2.5 A diagram illustrating the operation of a back extrusion fixture being used to measure the textural properties of a flowable food material.

4. Turn on the mechanical testing machine and attach the appropriate fixture (back extrusion cell).
5. Turn on the chart recorder or computer.

   If using computer software, procedures specific to the software will need to be followed.

6. For test equipment driven by computer software, enter required information such as specimen dimensions, cross-sectional area, desired calculations, and the rate at which the sample will be deformed (cross-head speed or deformation rate).
7. Place the specimen in the container (fill to about ¾ capacity) and mount on the lower plate of the mechanical testing machine.
8. Move the top fixture (i.e., the plunger) to a position near the top surface of the specimen. Measure the distance between the lower plate and the fixture to obtain the sample height, which may be required by computer software or in future calculations.
9. Zero the position of the fixture in relation to the sample surface and the load on the load cell.
10. For the back extrusion test, select a desired deformation rate.

    Testing should cease at the appropriate depth of compression of the packed sample.

11. Start the test.

    CAUTION: *The cross-head will begin to move down. Keep hands clear!*

12. Record the force/deformation data for the test.

    The rupture force may or may not be the represented as the maximum point on the force/deformation curve (see Figure H2.2.2). At rupture the food material begins to flow back up through the annular gap.

COMMENTARY

Background Information

deMan (1976) defined texture as "the way in which the structural components of a food are arranged in a micro and macro structure and the external manifestations of this structure." The external manifestations of structure refer to the behavior of a food in a mechanical testing machine. Texture can be subdivided into two categories: rheology or physical qualities (e.g., Young's modulus, Poisson's ratio) and haptaesthesis or perceived qualities (e.g., mouthfeel, gumminess; Bourne, 1982). These qualities can be measured using a variety of different instruments.

There are four main procedures for measuring the texture of food: empirical, imitative, fundamental, and ideal (Bourne, 1982). Of these procedures, empirical tests are the most frequently used in textural measurement; however, they are poorly defined (Bourne, 1982). The protocols provided in this unit are all empirical tests.

Perceived qualities of texture require an understanding of the way in which humans interact with food (Bourne, 1982). Mastication is the process of chewing and grinding food so that it may be swallowed. Humans masticate for flavor release, contentment, and to break food into smaller pieces (Bourne, 1982). Texture can also be sensed by using the hand and fingers. Squeezing a food material between the thumb and forefinger is an excellent method for evaluating texture (Bourne, 1982).

Cone penetrometers

The cone penetrometer was first developed for measuring the firmness or yield point of solid fats such as butter or margarine (Bourne, 1982). Assuming that the cone angle, mass of the cone assembly, temperature, and time for penetration are held constant, results from cone penetrometry can be interpreted based on the depth of penetration (Dixon and Parekh, 1979). Dixon and Parekh (1979) applied a cone penetrometer for measuring the firmness of butter.

The properties of the test specimen, the weight and height of the cone, and the cone angle are determinants for the depth of pene-

tration of a cone penetrometer (van Vliet, 1999). Should the specimen yield, elongational flow and shear flow results (van Vliet, 1999).

Puncture probes

Puncture probe testing involves measuring the maximum force required to drive a metal probe into a structured food (deMan, 1976). On a force/deformation curve, the force at rupture is seen as the maximum point on the curve. These instruments can be hand operated or attached to a force/deformation measuring instrument (e.g., mechanical testing machine). Puncture probes may have either a flat or a rounded end (Bourne, 1979). Puncture probes of the Magness-Taylor type are often used for firmness measurements of fruits and vegetables. Lurie and Nussinovitch (1996) found that a good correlation existed between the Magness-Taylor measurement of firmness and the compression testing of both strength and stiffness of apple cultivars. In firm apples, compression testing appeared to provide results most closely related to the properties perceived by humans (Lurie and Nussinovitch, 1996). Whether a puncture-type test or a compression-type test is more appropriate for testing texture may depend on the nature of the food.

The yield force reading obtained from a puncture test was found to depend on the perimeter and area of the punch (Bourne, 1979), as illustrated in the equation (Bourne, 1982):

$$F = K_c A + K_s p + c$$

where K_c and K_s are compression and shear coefficients, respectively, p is the perimeter of the sample, and c is a constant. K_c can be determined as the slope of a force/area curve, and K_s can be determined from the slope of a force/perimeter curve. It has been demonstrated that if the perimeter of the puncture probe is held constant, the force on a material is directly proportional to the area of the punch (Bourne, 1979). When the area of the punch is held constant, the force is directly proportional to the perimeter of the punch (Bourne, 1979). In cases where the puncture probe has a circular cross-section, the area and perimeter terms are replaced by diameter.

Of great importance, when using a hand-held puncture probe, is the type of product being examined. Bourne (1982) described three categories for food materials. Products such as freshly harvested apples require a continual increase in force to continue penetration after the yield point. However, food materials such as ripe fruit require no such increase, and food materials such as raw vegetables require pushing the puncture probe into the material until it contacts the splash collar on the instrument.

Cutting instruments

For conducting shear testing, as in cutting a food sample, the Warner-Bratzler fixture is commonly used. The Warner-Bratzler blade has a V-shaped notch cut from it (Bourne, 1982). Warner-Bratzler fixtures are most often employed for measuring the tenderness or toughness of meat products. To measure this property, the blade forces the meat into the V notch until it is cut though (Bourne, 1982). As the specimen fills the area of the V notch, it is cut or sheared; however, it should be noted that the stresses induced in this action are complex, involving tensile and compressive stresses as well as shear.

Normally, meat samples are oriented perpendicular to the blade (Voisey, 1976). This allows for measurement of the force required to cut across the muscle fibres. Cylindrical specimens ranging in size from 0.5 to 2.5 cm in diameter are most often used (Voisey, 1976). A true Warner-Bratzler test requires a deformation rate of 22.86 cm/min (Voisey, 1976).

Stanley (1976) found that, for a Warner-Bratzler shear cell, the firmness of a meat product may be reflected by measurements in compression, whereas tenderness may be reflected by the tensile strength of the meat. He further indicated that firmness and tenderness may or may not be dependent on each other. For this reason, results from such tests may be difficult to interpret.

The Kramer Shear Press can be equipped with test fixtures designed to measure shearing force. It was developed originally for testing the tenderness or toughness of peas (Stanley, 1976). The multiblade Kramer shear cell consists of ten blades that are driven down through a sample (Stanley, 1976). The sample rests in a box with slits on the bottom. As the blades enter the box, the sample is compressed, sheared, and extruded through the bottom of the box (Rao, 1992). Food materials are cut into cubes or chunks and the test cell is filled to half capacity (Voisey, 1977). According to Voisey, failure occurs in two manners; by compression and by cutting. The deformation required for failure depends on the resistance of the specimen to compression and cutting.

Structured foods, such as cheddar cheese and some fruits and vegetables, are often examined using wire cutting fixtures known as sec-

tilometers. In this case, a steel wire is moved through a sample at a controlled rate and the force on the wire is measured (deMan, 1976). Multiwire sectilometers are sometimes used to improve the precision of the testing procedure (Voisey and deMan, 1976).

Compression-extrusion fixtures

Finally, compression-extrusion testing involves an extrusion cell commonly used for weakly structured, homogeneous food products. This apparatus consists of a piston that is forced into a cylinder open at one end and containing the product (Figure H2.2.5). Beyond the point of rupture of the food, the compressed material is forced to flow back through the annular space between the piston and the cylinder (Bourne, 1976; Edwards, 1999). The gap between the piston and the cylinder is called the annulus (Bourne, 1982). Variation in the annulus width results in variation in the force required for extrusion (Bourne, 1982).

Anticipated Results

Textural measurements using a puncture probe should show a point on the force/deformation diagram where the force is a maximum. The maximum force encountered by the puncture probe, for a given sample, will vary depending on the geometry and size of the probe and the deformation rate. Cone penetrometer measurements will vary depending on the mass and angle of the cone, as well as the nature of the sample and deformation rate. Cutting instruments (i.e., Warner-Bratzler, Kramer shear, sectilometer) all make measurements based on the maximum force encountered by the fixture. The maximum force depends on the nature of the sample (e.g., cheese versus butter for a sectilometer) as well as deformation rate. Note that variable deformation rate is not a factor with Warner-Bratzler tests. Finally, the back extrusion fixture results should show a point of rupture for the food material, beyond which the food should be extruded. This point will depend on the nature of the sample, the deformation rate, the width of the annular gap, and the geometry of the plunger.

Time Considerations

Sample preparation may require as little as 5 min (e.g., removing the skin from an apple) or up to an hour or more (e.g., preparation of a food polymer solution and pouring into a mold). The time to run a test will depend on the deformation rate and the desired amount of penetration into the food material. Typically, a test using any of these special fixtures can be accomplished in 5 to 20 min.

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The editorial board is grateful to Dr. Ian Britt (University of Guelph) for his assistance in preparing this contribution.

Texture Profile Analysis

This unit describes an instrumental texture profile analysis (TPA) using mechanical compression of a foodstuff. TPA was developed in the early 1960s to study the mechanical properties of foods and their relationship to the texture of foods. The original TPA was carried out using the General Foods (GF) Texturometer by Friedman et al. (1963). A newer TPA was developed using the Instron Universal Testing Machine by Bourne (1968) with the following testing conditions: (1) a circular plate 150 mm in diameter was used to compress the specimens, so that samples were not subjected to any shearing; (2) the plunger was set to compress the 10-mm sample to 2.5 mm (25% of its original height), then rise back to its starting position and move down again to compress the sample to 2.5 mm (25% of its original height); and (3) the Instron cross-head was set to cycle with a vertical reciprocating movement at a constant speed of 50 mm/min.

Currently, there are many compressive instruments that are suitable for TPA. In the following protocols, the Instron Universal Testing Machine is used. In the Basic Protocol, the textures of vegetables, both raw and cooked to different temperatures, are measured. For Alternate Protocol 1, the textures of different fresh apple cultivars are tested. The texture of cooked ground beef samples with varying fat content is measured in Alternate Protocol 2. Lastly, in Alternate Protocol 3, gel textures are tested among samples with varying gelatin concentrations.

MEASURING THE TEXTURE OF POTATOES AND CARROTS

BASIC PROTOCOL

Texture is an important characteristic of vegetables, and it changes during thermal processing due to the breakdown of cellular material. Although both potato and carrot are root plants, the potato has a relatively uniform granular tissue structure and high starch composition, while the carrot has a fibrous tissue structure and low starch composition. These structural and compositional differences can affect the textural changes of potatoes and carrots with thermal processing.

Materials

Eight potatoes of uniform size (~50-mm square-mesh grading)
Eight carrots of uniform size (~280 mm long and 125 mm thick at the larger end)

Cork borer, size 4
Sharp knife
Instron Universal Testing Machine (Instron)

1. Set aside a potato and a carrot to use as a raw standard.
2. Cook each of the remaining seven samples of potatoes and carrots to an internal temperature of 30°, 40°, 50°, 60°, 70°, 80°, and 90°C.

   A calibrated digital cooking thermometer is preferable for measuring sample temperature.
3. Collect from each sample (including the raw standards) four cylindrical specimens measuring 10 mm high and 12 mm in diameter using a cork borer and a sharp knife.
4. Set an Instron Universal Testing Machine to compress the specimen twice, compressing each time to 50% of its original height.
5. Test specimens at 10 mm/min cross-head speed at room temperature (~22°C).
6. Analyze the four specimens taken from each sample at each cooked temperature and raw state.
7. Obtain TPA parameters for each sample (see Commentary).

Contributed by Yoshi Mochizuki

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ALTERNATE PROTOCOL 1

MEASURING THE TEXTURE OF APPLES

Apple cultivars have different textures due to their internal variability of structure and composition. Some apples resist boiling and do not readily sauce. Others may undergo ready cell separation. This wide range of textural behavior illustrates the complexity due to pectins and other cell-wall materials. Select apple cultivars according to the desired processing qualities.

Additional Materials *(also see Basic Protocol)*

Mature apple for each cultivar to be tested (all of a similar size, age, and ripeness)
Cork borer, size 6

1. Slice an apple at ~15 mm above and below its equator.
2. Collect five cylindrical specimens measuring 10 mm high and 15 mm in diameter from the central slice using a cork borer and a sharp knife. Avoid the core region.
3. Set an Instron Universal Testing Machine to compress the specimen twice, compressing each time to 80% of its initial height.
4. Test specimens at 50 mm/min cross-head speed at room temperature (~22°C).
5. Analyze the five specimens taken from each cultivar.
6. Obtain TPA parameters for each cultivar (see Commentary).

ALTERNATE PROTOCOL 2

MEASURING THE TEXTURE OF COOKED GROUND BEEF

Ground beef has a typical fat level of 20% to 30%. Consumers can select retail ground beef with decreased levels of fat; however, they usually perceive leaner grinds as being less palatable. To some extent this is true. In order to assure qualities such as texture, mouthfeel, tenderness, juiciness, flavor, appearance, and overall acceptability, a certain fat content is necessary in ground beef. The fat level can affect the texture of cooked ground-beef patties.

Additional Materials *(also see Basic Protocol)*

Ground beef of various fat content (~250 g of each)
Electric skillet, preheated to 150°C

1. For each type of ground beef, form two uniform patties (~113 g/patty) measuring 15 mm high and 60 mm in diameter.
2. Cook patties to an internal temperature of 77°C using a preheated (150°C) electric skillet. Cool to room temperature.

   A calibrated digital cooking thermometer is preferable for measuring sample temperature.
3. Cut four specimens as slices (10 cm × 30 cm × 10 mm) from each cooked patty.
4. Set an Instron Universal Testing Machine to compress the specimen twice, compressing each time to 30% of its initial height.
5. Test specimens at 200 mm/min cross-head speed at room temperature (~22°C).
6. Make eight measurements for each type of ground beef (four slices from each of the two cooked patties).
7. Obtain TPA parameters for each type of ground beef (see Commentary).

MEASURING THE TEXTURE OF GELATIN GEL

ALTERNATE PROTOCOL 3

In the food industry, particularly the confectionery industry, gelatin is commonly used for processing gelled products. Gelatin is a soluble polypeptide derived from insoluble collagen, and it shows a reversible sol-gel change with temperature. The rigidity of the gel is one of the most important properties of gelatin and correlates with the gelatin concentration. In this protocol, the gelling quality of a gelatin is measured by measuring the gel strength as a function of gelatin concentration.

Additional Materials *(also see Basic Protocol)*

Gelatin
19 × 31–cm enamel pans, >2 cm in depth
Wire-cutting grid to cut 2-cm cubes

1. Mix 22, 24, 28, 35, and 45 g gelatin in five separate beakers each containing 1 liter boiling water and stir 10 min so that gelatin is dissolved.
2. Cool samples to 40°C and pour them into 19 × 31–cm enamel pans to a height of 2 cm.
3. Cover enamel pans with aluminum foil and refrigerate 24 hr at 10°C.
4. Cut three 2-cm cubes from each concentration of gelatin gel using a wire-cutting grid.
5. Set an Instron Universal Testing Machine to compress the specimen twice, compressing each time to 30% of its initial height.
6. Test specimens at 200 mm/min cross-head speed at room temperature (~22°C).
7. Analyze the three specimens taken from each gelatin concentration.
8. Obtain TPA parameters for each gelatin concentration (see Commentary).

COMMENTARY

Background Information

The instrumental TPA was developed by a group at the General Foods Corporation Technical Center (Szczesniak and Kleyn, 1963). The parameters obtained from the resulting force-time curve correlate well with sensory evaluations of the same parameters (Friedman et al., 1963). Later, the Instron Universal Testing Machine was adapted to perform a modified TPA (Bourne, 1968, 1974). A typical Instron TPA curve is shown in Figure H2.3.1.

The difference between the GF Texturometer and the Instron arises from the different compression motions. The plunger of the GF Texturometer is driven at sinusoidal speed and follows the arc of a circle. The resulting TPA curve shows rounded peaks. The plunger of the Instron, however, is driven at constant speed and shows rectilinear-shaped peaks on its TPA curve. Therefore, one disadvantage of the GF Texturometer is that the contact area between the sample and the plunger is not constant during the test; because the plunger moves through the arc of a circle, one edge of the plunger contacts the food first and, as the downstroke continues, the area of the plunger pressing on the food increases until the entire plunger area is in contact with the food at the end of the downstroke (Bourne, 1968). Another disadvantage of the GF Texturometer is that the sample platform is flexible and bends a little as the load is applied. This can result in confounding data. On the other hand, the Instron is so rigid that bending of the instrument does not occur, resulting in data that are precise. All modern TPA measurements are now carried out with two-cycle uniaxial compression instruments such as the Instron Universal Testing Machine.

The most common parameters derived from the TPA curve are shown in Table H2.3.1 (Friedman et al., 1963; Bourne, 1968). The peak force during the first compression cycle is defined as hardness. Fracturability (originally called brittleness) is defined as the force at the first significant break in the curve during the first compression cycle. The ratio of the positive force area during the second compression cycle to that during the first compression

cycle (area 2/area 1; Figure H2.3.1) was originally defined as cohesiveness when using the GF Texturometer. When the Instron is used, cohesiveness is obtained from the areas under the compression portion (downstroke) only and excludes the areas under the decompression portion (upstroke) instead of using the total area under positive force. The negative force area of the first compression cycle (area 3), adhesiveness, is defined as the work necessary to pull the plunger away from the sample. The length to which the sample recovers in height during the time that elapses between the end of the first compression cycle and the start of the second compression cycle is defined as springiness (originally called elasticity). Gumminess is defined as the product of hardness times cohesiveness, and chewiness is defined as the product of hardness times cohesiveness times springiness.

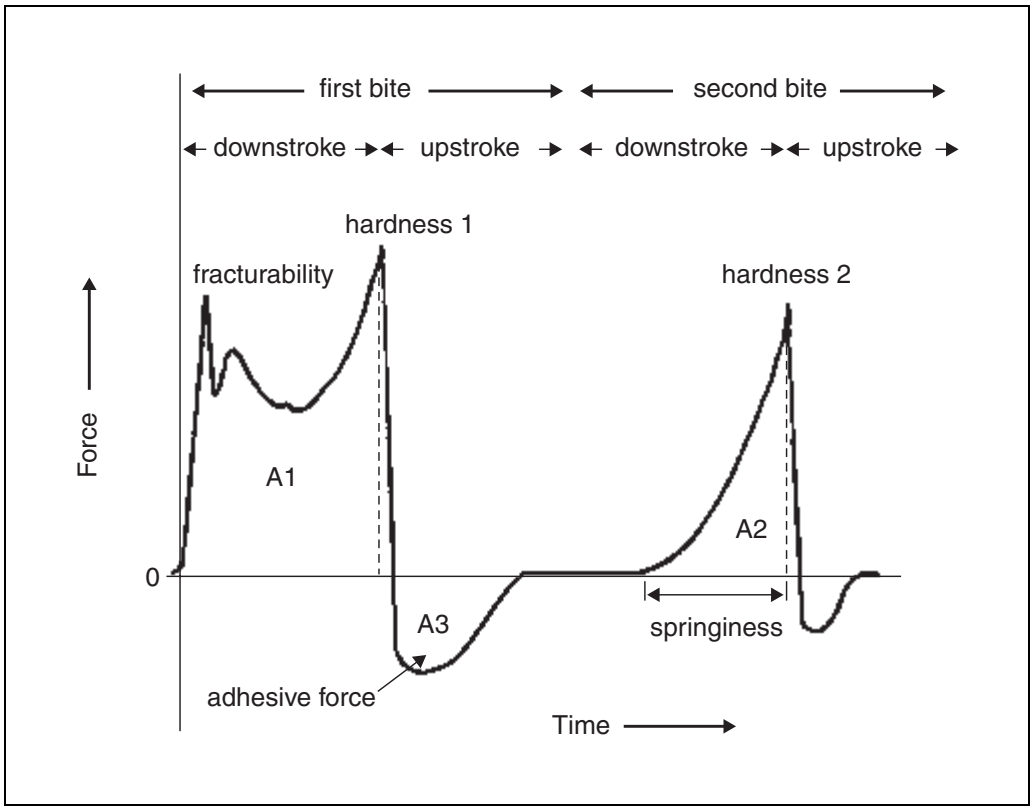

Figure H2.3.1 A generalized texture profile analysis curve from the Instron Universal Testing Machine. A1, area 1; A2, area 2; A3, area 3. The dotted lines indicate the times at which hardness is measured. See text for further discussion. Reprinted from Pons and Fiszman (1996) with permission from Food and Nutrition Press.

Table H2.3.1 Parameters and Units of Instrumental Texture Profile Analysis[a]

Mechanical parameter	Measured variable	S.I. units
Adhesiveness	Work	N × mm (mJ)
Chewiness	Work	N × mm (mJ)
Cohesiveness	Ratio of forces	Dimensionless
Fracturability	Force	N
Gumminess	Force	N
Hardness	Force	N
Springiness	Distance	mm

[a]From Bourne, 1982.

Critical Parameters

Consistent preparation of samples is probably the most critical step in obtaining good data. Another important step is the cutting and slicing of test samples from the prepared samples. Having the same size for each test sample is very critical. Generally, it is also important to avoid edge effects in a test sample. For example, for cooked samples, a test sample taken from an exterior surface will generally give different readings than one taken from the interior. Unless otherwise specified, avoid taking test samples close to the exterior surfaces of prepared samples. It is also important to have consistent aging effects on the texture of prepared samples between the time they are prepared and tested.

For a TPA measurement, make sure that the degree of compression, plunger size, and cross-head speed are the same among tests that are to be compared. Adjust the operating characteristics of the machine when testing other types of samples. For example, if the samples are harder than those used in these protocols, reduce the degree of compression and/or the cross-head speed.

Anticipated Results

Tables H2.3.2 and H2.3.3 show TPA parameters of raw and cooked potatoes and carrots prepared at some of the temperatures described in the Basic Protocol. In general, hardness, fracturability, cohesiveness, springiness, gumminess, and chewiness decreased with increasing cooking temperatures. Thus, the major changes of texture during thermal processing were due to the breakdown of cellular material. These results suggest that TPA parameters are suitable to monitor the cooking of potatoes and carrots (Mittal, 1994).

Table H2.3.4 shows an example of cohesiveness data from apple cultivars as assayed in Alternate Protocol 1. The cohesiveness discriminates between Golden Delicious and Stark Delicious. The apples of extra quality showed the lowest values of cohesiveness. These results suggest that TPA parameters can differentiate between apple cultivars (Paoletti et al., 1993).

In Table H2.3.5, hardness, springiness, and cohesiveness of cooked ground beef patties as measured in Alternate Protocol 2 are given. The hardness for 5% and 10% fat levels was higher than for 15% or higher fat levels. Beef patties containing 25% and 30% fat had lower springiness values than all other patties. The cohesiveness for beef patties containing 5% fat was higher than others. However, cooking temperature had no effect on springiness and cohesiveness (Troutt et al., 1992).

Figure H2.3.2 shows the maximum and yield force (i.e., hardness and fracturability) of

Table H2.3.2 Changes in the Texture of Potatoes Cooked to Various Center Temperatures[a]

Temperature (°C)	Chewiness (mJ)	Cohesiveness	Fracturability (N)	Gumminess (N)	Hardness (N)	Springiness (mm)
20	38.5	0.087	107	12.6	145	3.05
40	29.4	0.071	104	10.5	148	2.81
60	16.2	0.049	121	6.6	135	2.45
80	2.8	0.047	26	1.4	37	2.10

[a]From Mittal, 1994.

Table H2.3.3 Changes in the Texture of Carrots Cooked to Various Center Temperatures[a]

Temperature (°C)	Chewiness (mJ)	Cohesiveness	Gumminess (N)	Hardness (N)	Springiness (mm)
20	73.7	0.104	22.3	215	3.30
40	39.6	0.067	15.3	228	2.59
60	22.0	0.048	8.9	186	2.46
80	16.0	0.065	5.9	91	2.69

[a]From Mittal, 1994.

Table H2.3.4 Cohesiveness Values from TPA Measurements of Different Apple Cultivars[a]

Apple cultivar	Cohesiveness
Annurea	0.0192
Emperor	0.0171
Golden Delicious Extra	0.0135
Golden Delicious I	0.0299
Granny Smith	0.0520
Renetta	0.0090
Stark Delicious Extra	0.0156
Stark Delicious I	0.0205

[a]From Paoletti et al., 1993.

Table H2.3.5 TPA Values of Cooked Ground-Beef Patties with Different Fat Levels[a]

TPA parameter	Fat level (%)					
	5	10	15	20	25	30
Cohesiveness (%)	47.5	41.0	37.8	38.0	34.5	36.0
Hardness (N)	.96	.84	.69	.59	.59	.52
Springiness[b]	86.2	85.8	82.9	82.2	77.9	78.1

[a]From Troutt et al., 1992.
[b]Springiness was calculated as a ratio.

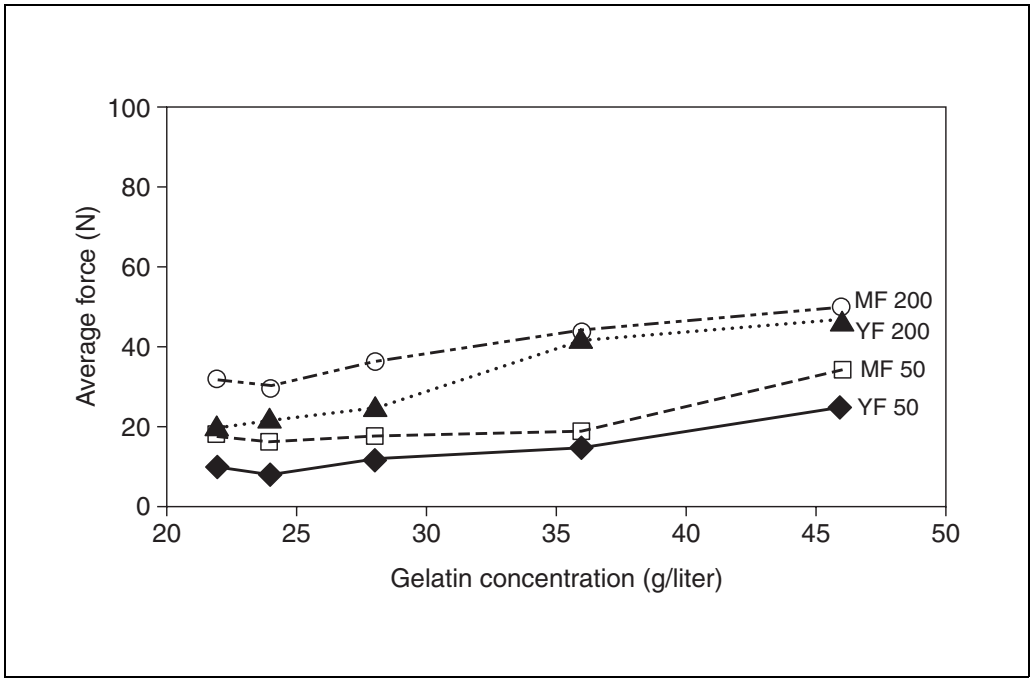

Figure H2.3.2 Average maximum and yield force (i.e., hardness and fracturability) of gelatin gels at different concentrations. Six samples for each concentration were analyzed under compression to 85% with a flat-plate probe. MF, maximum force at 50 and 200 mm/min; YF, yield force at 50 and 200 mm/min. Reprinted from Muñoz et al. (1986) with permission from Food and Nutrition Press.

gelatin gels at different concentrations. The results show that hardness and fracturability increased as the gelatin concentration increased. However, the values of hardness and fracturability were dependent on the crosshead speed. Also, the cohesiveness for gelatin gels was not significantly correlated with the concentration of gelatin (Muñoz et al., 1986).

Limitations of the instrumental TPA when compared to sensory TPA include sensitivity and complexity. However, the instrumental TPA parameters are repeatable. Also, TPA parameters correlate well within sensory TPA parameters. The best correlation and data greatly depend on the measuring conditions, the plunger, the precise sample shape, and, most importantly, the careful and skillful operation of the experimenter.

Time Considerations

The time required for one TPA test of a sample is ~5 to 10 min, which includes sample positioning and instrument cleaning. It is usually the case that sample preparation (e.g., cooking) requires much more time than TPA measurements. Most uniaxial compression instruments offered to the food industry are computer controlled and have software to immediately calculate the TPA parameters.

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Breene, W.M. 1975. Application of texture profile analysis to instrumental food texture evaluation. *J. Texture Stud.* 6:53-82.

Reviews developments and applications of the instrumental TPA using the GF Texturometer and the Instron.

Bourne, 1982. See above.

Describes food texture and viscosity including the definition of TPA characteristics using the GF Texturometer and the Instron.

Pons and Fiszman, 1996. See above.

Reviews testing conditions and TPA terminology of the instrumental TPA.

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Dynamic or Oscillatory Testing of Complex Fluids

Real world materials are not simple liquids or solids but are complex systems that can exhibit both liquid-like and solid-like behavior. This mixed response is known as viscoelasticity. Often the apparent dominance of elasticity or viscosity in a sample will be affected by the temperature or the time period of testing. Flow tests can derive viscosity values for complex fluids, but they shed light upon an elastic response only if a measure is made of normal stresses generated during shear. Creep tests can derive the contribution of elasticity in a sample response, and such tests are used in conjunction with dynamic testing to quantify viscoelastic behavior.

This unit describes four procedures for evaluating the viscoelastic properties of complex foods. These procedures should be used for samples with unknown viscoelastic properties. The instrument used for this analysis is a rotational rheometer, which is described in detail in *UNITS H1.1 & H1.2*. References to rheometers that are commercially available can be found in Schoff and Kamarchik (1996). To measure viscosity, a steady shear stress or shear rate is applied, and the sample response is measured in terms of shear rate or shear stress, respectively. A dynamic or oscillatory test is the most sensitive measure of viscoelastic structure; the sample is perturbed with a sinusoidal input (stress or strain), and the sinusoidal response is measured. The relative amplitudes of the two signals and their phase difference (δ; Barnes et al., 1989) are used to calculate modulus and viscosity (Figure H3.1.1). Thus the ratio of stress/strain defines the modulus (G) at a given moment. These parameters are complex, however, because of the sinusoidal nature of the test, and each one may be resolved (i.e., split mathe-

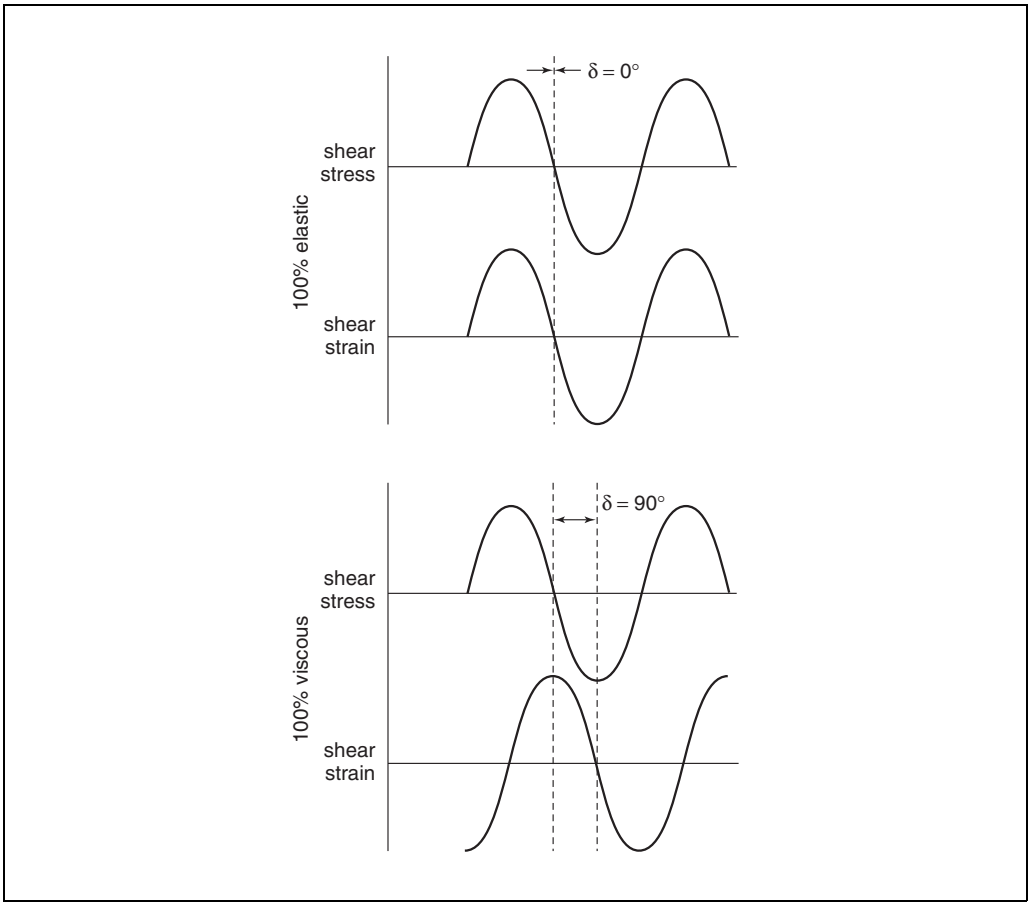

Figure H3.1.1 Responses from purely elastic or purely viscous foods under oscillatory testing. The controlled parameters of the oscillatory applied stress or strain are amplitude, frequency, temperature, and duration of measurement. The measured parameters are amplitude and phase shift (δ) of the strain or stress response.

Contributed by Peter Whittingstall
Current Protocols in Food Analytical Chemistry (2003) H3.2.1-H3.2.11

matically) into an in-phase and an out-of-phase component. The mathematics of this process are similar to vector analysis, where a single vector is resolved into two components at right angles to each other (Figure H3.1.2). From the pythagorean theorem:

$$G^* = \sqrt{(G')^2 + (G'')^2} = \frac{\text{stress}}{\text{strain}}$$

The complex modulus, G^*, is resolved into a real component, G', and an imaginary component, G'':

$$G^* = G' + iG''$$

where i is the square root of −1. The real component (G') is completely in phase. It represents pure elasticity or energy storage, and is thus called the storage modulus. The remainder of the signal (G'') is considered out of phase and imaginary in terms of the elasticity. The energy that is not stored must be dissipated or lost, and thus G'' is called the loss modulus, representing energy lost through viscous dissipation. It is not, however, a measure of viscosity. For that, the complex viscosity, η^*, is used, which also comprises two components, η' (dynamic viscosity) and η'' (which is not normally named, but represents undissipated energy and thus is connected to elasticity):

$$\eta^* = \eta' - i\eta''$$

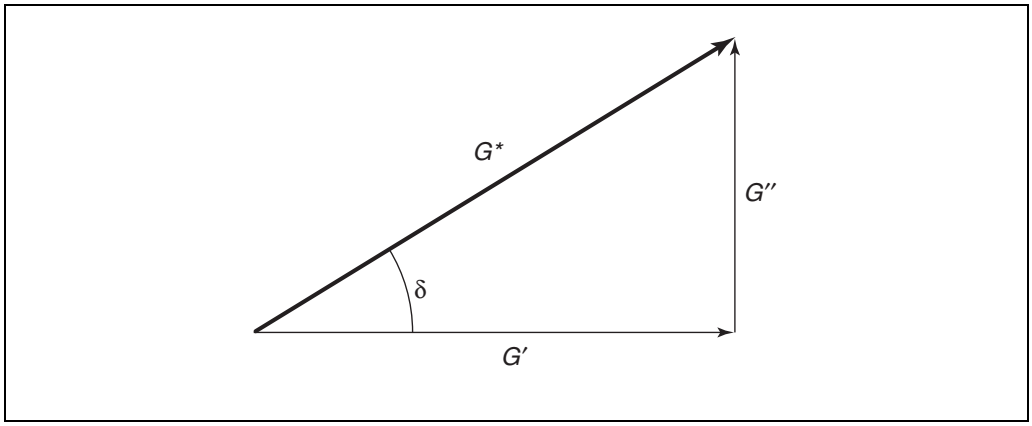

Figure H3.1.2 The oscillatory response of a food material that possesses both elastic and viscous properties can be represented by a complex variable G^*. This variable has two components that can be expressed as either the Cartesian coordinates G' and G'' or the polar coordinates $|G^*|$ and δ. $|G^*|$ is the magnitude of the imaginary G^* and is measured as the ratio of the amplitudes of stress and strain.

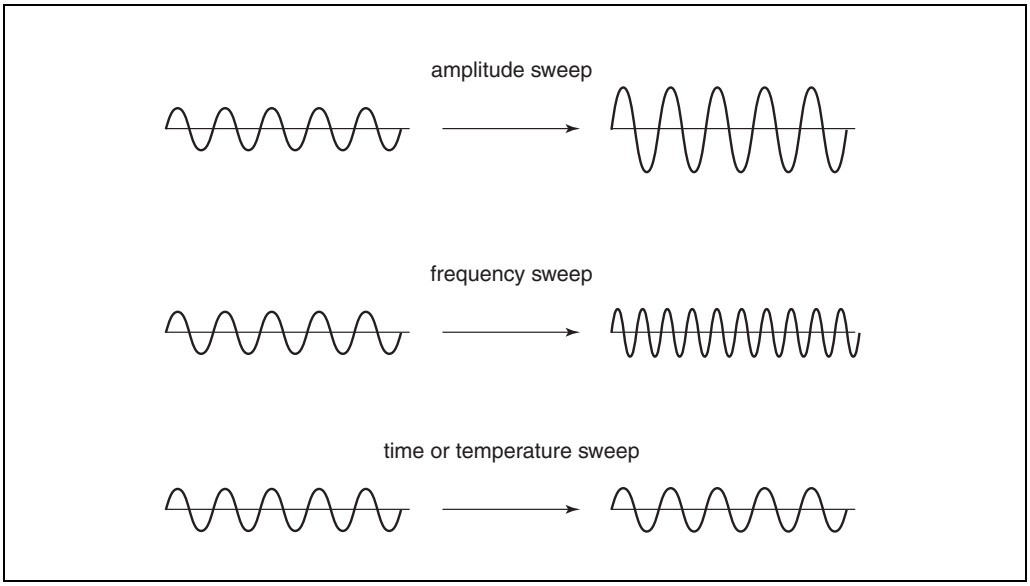

Figure H3.1.3 For oscillatory (sweep) testing, four control parameters can be varied: amplitude, frequency, time, and temperature.

For a complete evaluation of an unknown sample, all four of the following procedures should be performed in the order presented. First, it must be determined whether the sample is at steady state before rheological measurements are undertaken. Second, the linear viscoelastic (LVE) region must be determined. Third, a mechanical spectrum (or fingerprint) of the sample must be obtained using the frequency sweep test. Finally, changes in structure and chemorheology should be evaluated using a temperature sweep test. These procedures require a significant amount of common methodology that has been described in the Strategic Planning section of *UNIT H1.2*. For some samples that have strong solid-like behavior, special preparation is required, the nature of which will depend on the specific sample being analyzed (e.g., gels in *UNIT H3.2*). For the purpose of this discussion, it is assumed that all necessary choices have been made in terms of geometry selection, and that the rheometer has been set up to accept the sample for testing. Schematics of the various test types are shown in Figure H1.1.1. Schematics of the various test variables are shown in Figure H3.1.3. For general rheometry artifacts, see *UNIT H1.2*. Common artifacts such as sample drying, aging, or structure rebuilding are best detected using Basic Protocol 1 of that unit.

PROCEDURE 1: DETERMINING IF A SAMPLE IS AT STEADY STATE

The use of the dynamic (oscillation) time sweep (Figure H3.1.3) is used to monitor changes in viscoelastic structure. In Figure H3.1.4, a grease sample is used as an example of a slow recovery process.

The sample is loaded onto the instrument and the time reference is noted by starting a timer or resetting a timer in the software. A dynamic test for viscoelastic structure is then used to monitor changes in the sample that could result from mechanical relaxation, drying, or thixotropy. A time sweep test is usually performed at a constant temperature. The test is also run at a constant frequency that is comparable to real-time observation (typically 1 Hz) or at a constant angular frequency (10 rad/sec or 1.6 Hz).

The amplitude of stress or strain is held constant at a value that is within the LVE region of the sample. For an unknown material, it can be difficult to determine a reasonable value to use. If the sample is changing with time, however, it is impractical to measure the extent of the linear region until a steady state is reached. To overcome this, a controlled strain amplitude of 0.1% to 1.0% should be set before starting the test. The rheometer will operate directly if it is a controlled-strain instrument or through a feedback loop if it is a controlled-stress instrument. (*UNIT H1.1* discusses the differences between these two rheometer types.) The choice of the actual value is dictated by the type of sample. Polymer solutions and simple liquids can tolerate high strains, so ~1% strain can be used. Gels and pastes are more sensitive, so 0.1% to 0.5% strain should be used. If the stress signal generated by the transducer in a controlled-strain instrument is too low (i.e., in the range of noise), the sample must be re-evalu-

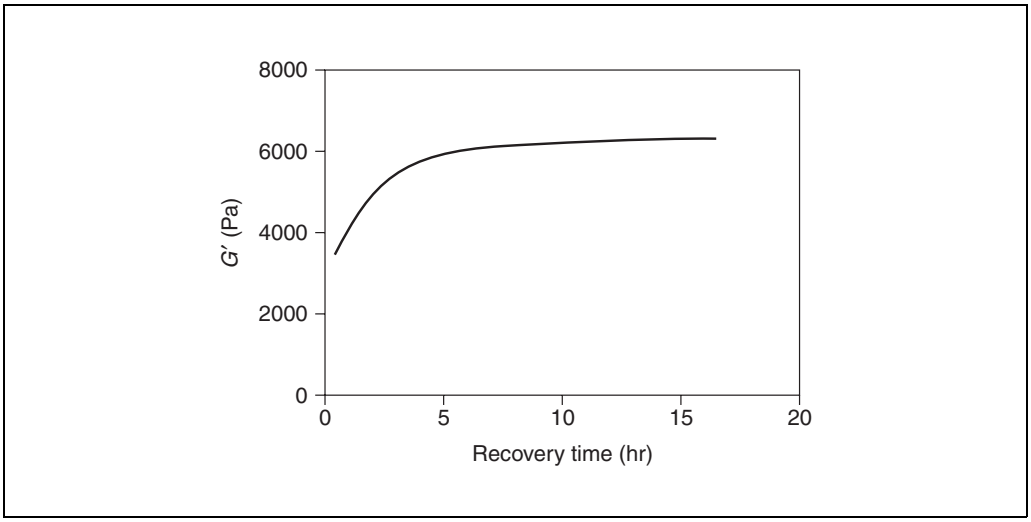

Figure H3.1.4 Structure recovery after loading a sample of a grease or fat on a cone-and-plate fixture on a rotational rheometer. *G′* was measured over a period of 17 hr at constant oscillatory stress, frequency, and temperature.

ated with a more sensitive transducer or a larger geometry.

There may be other settings in the test method, such as timings for data collection, and these should normally be left at the default values. The time for the experiment should typically be set to ~30 min. This is normally adequate, but if steady state occurs more quickly, it is not a problem, because most tests can be interrupted. The instrument should be set to display the following information in real time: storage modulus (G'), loss modulus (G''), corrected phase angle (δ), and the actual percent strain. If the instrument has the capability to display the waveforms visually, this should be switched on.

As the test progresses, the values of G' and, if possible, the stress required to achieve the commanded strain should be recorded. (These data should be used to design subsequent experiments so as to avoid exceeding the stress at which LVE behavior becomes non-linear.) When G' has reached a steady state, its value should be approximately constant (within ±5%). Beware of slow but consistent increases in G' (~1% per data point), as this may be indicative of thixotropic behavior, which will require hours for the system to reach steady state, or of slow consistent drying. The solvent trap cover should be used to eliminate the possibility of slow consistent drying in the experiment. During the test, the stress measured by the transducer, or used by the motor to achieve the commanded strain, should be recorded. This value is important in constructing the next test to evaluate the width of the LVE region. Once steady state is achieved, the test can be halted and the data saved.

PROCEDURE 2: DETERMINING THE LINEAR VISCOELASTIC REGION

In this experiment, the same frequency and temperature used in the determination of steady state (procedure 1) are used, and the method is otherwise modified for analysis of G' as a function of stress (for controlled-stress instruments) or strain (for controlled-strain instruments). It is generally better to ramp through the stress or strain in logarithmic intervals, although a linear ramp is acceptable. Using the value for the stress needed to achieve a given strain, construct the range as follows. If, for example, a stress of 5 Pa was used to attain 1% strain, the initial stress should be set at 1 Pa or 0.1 Pa (expected strain of ~0.2% or 0.02%, respectively). This is based on the assumption that the preceding test was performed in the linear region so that the stresses and strains are in proportion. If a logarithmic test is to be performed, it is convenient to use decades (0.1%, 1.0%, 10%). A controlled-strain instrument allows for this range to be set directly. The upper limit of stress or strain is ultimately determined by the sample itself, but two to three decades will typically exceed the LVE limit of most materials. Thus, 10% or 100% strain is normally the upper limit for even flexible polymer systems.

Figure H3.1.5 shows the LVE region as a function of strain for a fat product (shortening) at 10 rad/sec. The LVE region is extremely narrow; strains of 0.02% are outside the linear

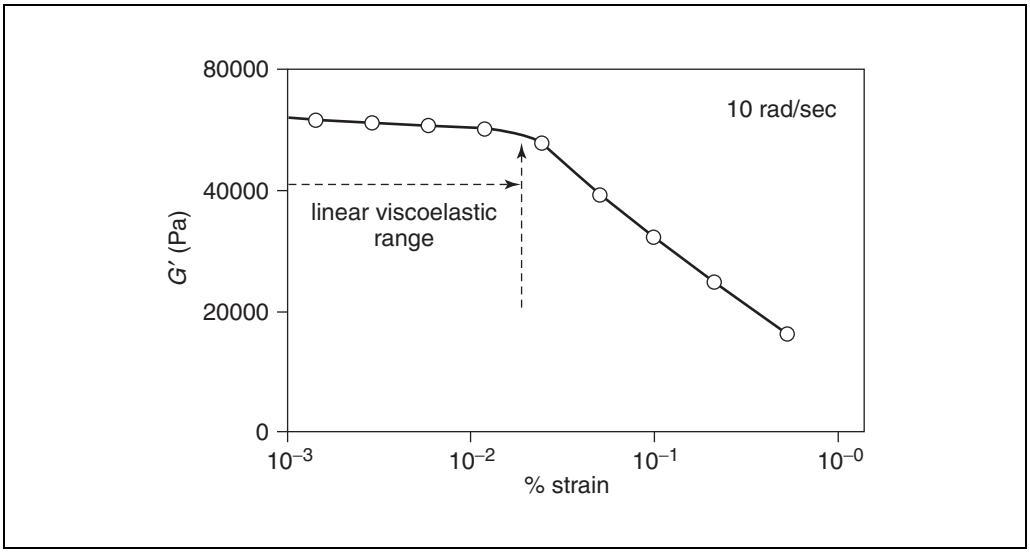

Figure H3.1.5 Determination of the linear viscoelastic range with respect to strain for a food shortening. The linear range appears to exist for strains of ≤0.02%.

zone. These data were acquired on a controlled-stress instrument and would probably be difficult to acquire on typical controlled-strain devices.

The same parameters should be displayed in real time to allow for visual determination of the point at which the linear region ends. Many materials will show a stable G' plateau, and the drop in G' that occurs beyond the LVE region is obvious. Some materials, especially highly filled ones, may always show a decline in G', and this decline is more pronounced in nonlinear behavior. Under such circumstances, it is usual to attribute the limit as occurring at $G' = 90\%(G'_{max})$. Other key indications of nonlinear behavior include a concomitant increase in G'' and δ as G' declines, as well as a change in the shape of the output signal waveform. This asymmetry is usually caused by an increase in the third harmonic in proportion to the fundamental.

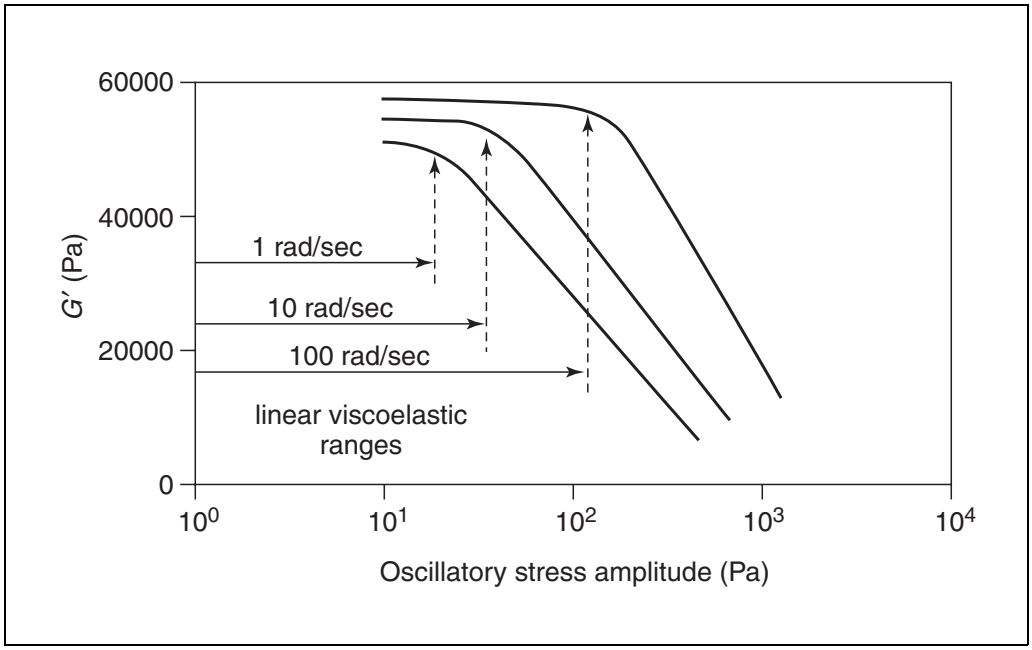

Figure H3.1.6 Dependence of the extent of the linear viscoelastic range of a food shortening on the frequency of the applied sinusoidal shear stress. As the stress frequency decreases, the range of linear behavior also decreases.

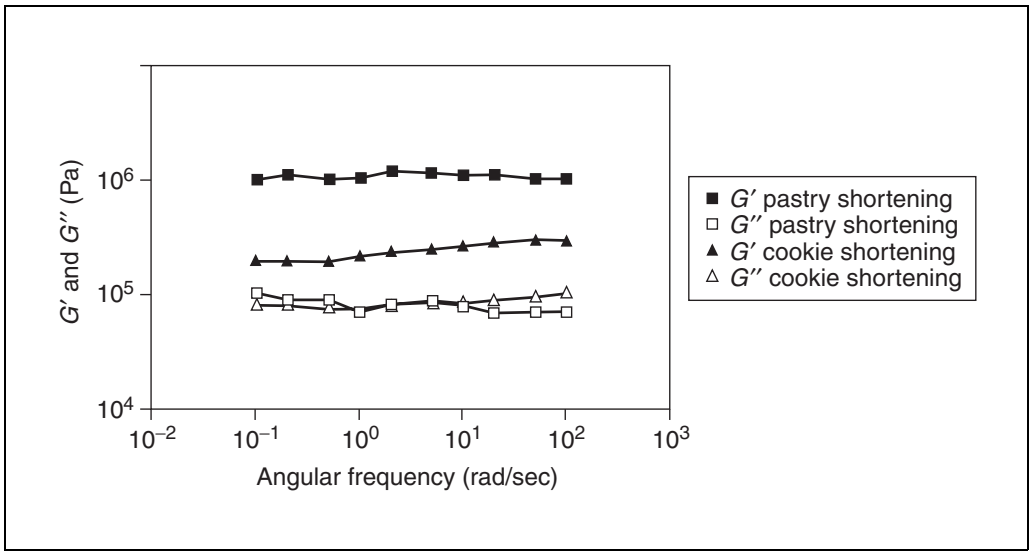

Figure H3.1.7 The use of a mechanical frequency spectrum to distinguish between cookie and pastry shortenings. Although both shortenings have similar G'' responses across different frequencies, their G' responses differ significantly.

Once a range of stresses or strains has been defined for linear behavior at one frequency, the method should be repeated at any other frequencies of interest. For example, if a frequency sweep test is to be run covering 0.1 to 100 rad/sec, then it makes good sense to determine the linearity at the lowest and highest frequencies to avoid obtaining nonlinear data in the test. Typically, a material will show a narrowing of linear behavior in terms of stress as the frequency decreases, but a broadening in terms of strain. Figure H3.1.6 shows the stress effect as a function of angular frequency for a shortening sample. At 1 rad/sec the linear region appears to end at ~15 Pa, at 10 rad/sec it ends at ~30 Pa, and at 100 rad/sec the end occurs at ~80 Pa.

PROCEDURE 3: OBTAINING A MECHANICAL SPECTRUM OR FINGERPRINT OF A SAMPLE

In the frequency sweep test, the idea is to obtain LVE data from the test material over the widest possible (or realistic) range of frequencies. The lower limit of testing is never difficult for a rheometer to achieve physically, but it may be impractical to explore. Typically, the time required to obtain data at frequencies of <0.01 rad/sec or 0.006 Hz is impractical for a laboratory schedule. (At 0.006 Hz, each data point would take 167 sec for a single iteration; most rheometers perform at least two or three iterations.) Furthermore, samples may change or degrade in nonsterile conditions over extremely long tests (i.e., hours). If it is desirable to obtain low-frequency data, the optimal approach may be to use alternative tests such as the creep test (for controlled-stress instruments) or the stress-relaxation test (for controlled-rate instruments), which are described elsewhere (Barnes et al., 1989). Figure H3.1.7 shows a comparison of two types of shortening. Both samples have the same type of response, but they can be distinguished from one another based on the significant difference in the magnitude of G'. In the figure, both samples appear to be predominantly elastic, as $G' > G''$, and insensitive to frequency. This is to be expected for a soft solid or gelled system.

The rationale behind a frequency sweep is to compare the behavior of a sample at short times (high frequencies) that mimic high shear rates with its behavior at long times (low frequencies) that mimic low shear rates. High shear is typically used to mimic food application (e.g., spreading mayonnaise) or processing, while low shear is typically used when assessing food stability. Another point to remember is that elastic behavior is favored at high frequency, whereas viscous behavior is favored at low frequency. Figure H3.1.8 shows a frequency sweep for an elastic liquid used in a soft drink, in which candy gel beads are suspended without sedimentation. At low frequencies, the values for G' reach a plateau. This is consistent with a sample that has an apparent yield stress. The steep rise in η^* at low frequencies also indicates that the system is stable and will be able to support the beads for prolonged periods.

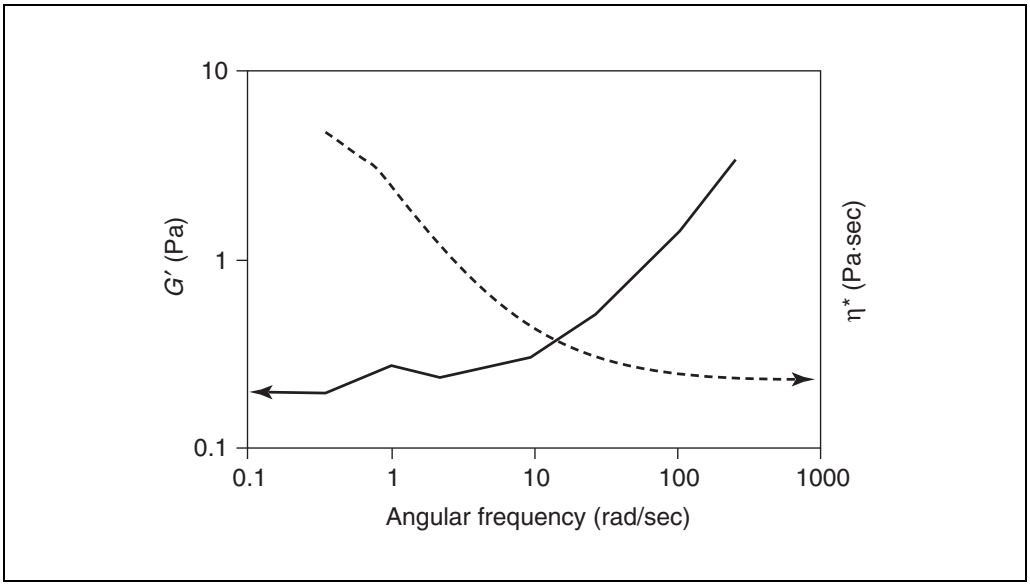

Figure H3.1.8 The characterization of a soft drink that shows a high complex viscosity, η^*, at low frequencies. This corresponds to a high viscosity at low shear rates, which gives the drink the ability to prevent the sedimentation of suspended gel beads. Solid line, G'; dashed line, η^*.

The frequency sweep can be performed at a constant stress or a constant strain amplitude, and care should be taken in selecting a value for either parameter. Viscoelastic materials, especially polymeric materials, may change their linear behavior quite drastically as a function of frequency. If the dynamic range of the rheometer has a limitation (e.g., stress transducer range in a controlled-strain instrument), then it may be feasible to test only two to three decades of frequency. At low frequencies the stress signal will drop dramatically for a given strain, presenting problems. In some sense the same issue is seen in a controlled-stress instrument at high frequencies, because the position (strain) data get very small for a given stress. To obtain wider ranges of data under these circumstances, the testing should be repeated at the new frequencies using different strains and stresses.

To cover the widest range of frequencies in a single test, a controlled-stress device operating in a controlled-strain mode, or the equivalent test for a controlled-strain device, will provide the optimal configuration because the stresses or strains, respectively, are boosted to stay within the dynamic range of the instrument. If the results for LVE behavior have not been generated, then a fixed strain of 0.1% to 1.0% is likely to be adequate. As before, lower stresses should be used if the sample is a dispersion or emulsion, and higher stresses should be used if it is polymeric in form.

Once a stress or strain has been selected, the range of frequencies is specified, usually in a logarithmic sense. Thus, if 10 rad/sec was used in the initial test to find the LVE region, then a frequency sweep from 1 to 100 rad/sec would be useful. A broader sweep of 0.1 to 1000 rad/sec (or the upper limit of the instrument) would give proportionately more information.

SPECIAL PROCEDURE: TIME-TEMPERATURE SUPERPOSITION

To obtain as much information as possible on a material, an empirical technique known as time-temperature superposition (TTS) is sometimes performed. This technique is applicable to polymeric (primarily amorphous) materials and is achieved by performing frequency sweeps at temperatures that differ by a few degrees. Each frequency sweep can then be shifted using software routines to form a single curve called a master curve. The usual method involves horizontal shifting, but a vertical shift may be employed as well. This method will not work for all materials. The method requires that the sample be thermorheologically simple, which means that its relaxation times change in a regular fashion as a function of temperature. Frequency sweep data are often analyzed using viscoelastic models. Generally, the parameters analyzed are G' and η', the in-phase components of elasticity and viscosity, respectively.

PROCEDURE 4: PROBING CHANGES IN STRUCTURE AND CHEMORHEOLOGY

The dynamic temperature sweep has many applications. First, it is used to follow changes in state, such as a melting phenomenon in chocolate, a glass transition in confectionary, or the baking process in a dough or batter. Second, it is used to follow irreversible gelation or curing in a sample. Many samples can be made to form a gel under the right conditions of temperature, and it is of great interest to follow the kinetics of structure buildup in real time, without disrupting the process itself. The latter test is normally done in a parallel-plate geometry, whereas the former could use cone-and-plate, parallel-plate, or concentric-cylinder geometries (Figure H1.1.1). Other examples include phase changes in lipid systems or emulsions.

A single frequency is traditionally used (1 Hz or 10 rad/sec) and a fixed strain or stress is applied. To obtain data points more rapidly it is advisable to use higher frequencies. The changes in structure can be very large, as many gels start out as weak liquids. For such samples, it is best to use a fixed strain (e.g., 0.1% to 1.0%). The duration of the test depends on the sample and the rate of the temperature ramp. It is important to ramp the temperature at a meaningful rate without introducing temperature gradients (normally, 1° to 5°C/min), with the lower ramp rates being preferred if run time is not a limitation. Figure H3.1.9 depicts the heating of cookie dough and its softening and then stiffening as it cooks.

Certain processes in a material are accelerated by increasing the temperature, such as phase separation in emulsions. A convenient way of predicting formulation stability is to run a temperature sweep and observe the response of G' or $\tan(\delta)$. Samples that are stable will tend to show almost no major change in these parameters, whereas unstable materials will change sharply at a critical temperature. Figure H3.1.10 shows comparative data for four emulsions that are divisible into two pairs: one pair that is likely to be stable and another that is

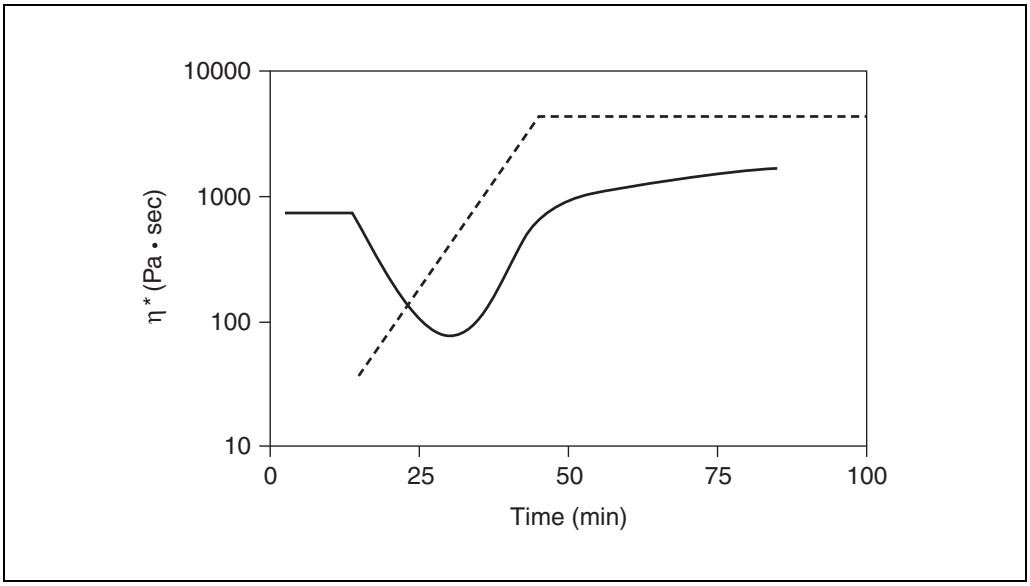

Figure H3.1.9 The characterization of the baking of a cookie dough by the measurement of the complex viscosity (η*, solid line) as a function of temperature (°C, dashed line).

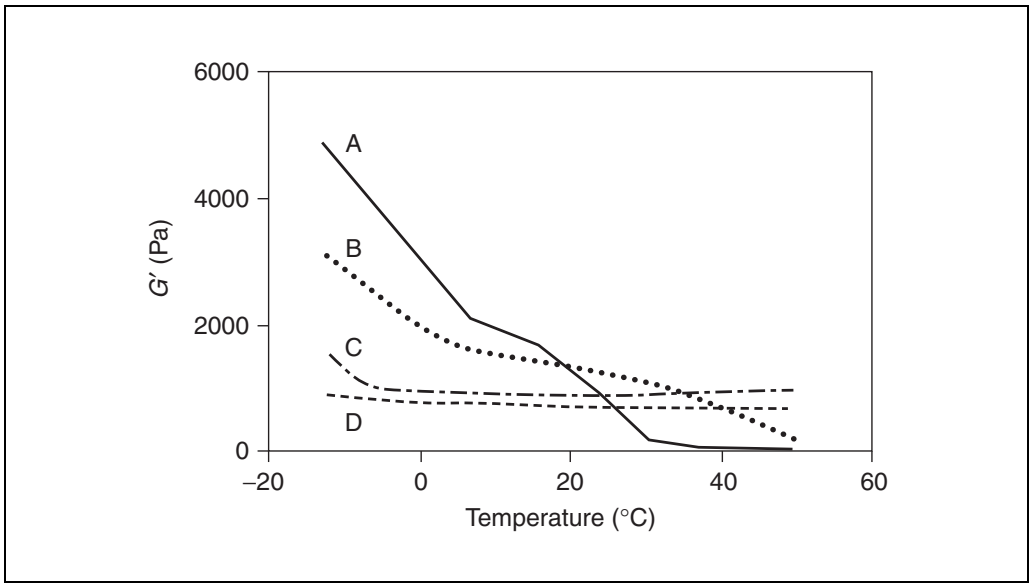

Figure H3.1.10 The temperature stability of four emulsions. Emulsions A and B show a high dependence of rheological stability on temperature. Emulsions C and D have rheological properties that are relatively independent of temperature.

likely to be unstable. Samples A and B clearly undergo transitions and lose elastic structure, whereas C and D are relatively unaffected. Consequently, it is easy to distinguish between the formulations to predict the more successful ones (i.e., C and D).

Another option is to use a multiwave technique, whereby the fundamental frequency and several other frequencies (harmonics) are added together into a single complex wave. Each one of these multiwave iterations can be deconvoluted into its components after the test is complete. Thus, each complex data point can be multiplied into several data points. Setting up such procedures involves assigning stresses or strains to each of the components, and there is a danger that the linear viscoelastic region of the sample will be exceeded because these stresses and strains may not add in a linear fashion. Often a series of preliminary experiments at each of the desired frequencies must be done in order to configure the multiwave test correctly. Because instruments vary in how they perform this type of procedure, it is beyond the scope of this work to describe this test in detail.

Because the sample may undergo significant changes in volume during the test, it is an advantage to use an instrument with an automatic feature to maintain a gap in response to the normal forces applied to the plates. Such a feature needs to be able to maintain a constant normal force within a window and to modify the gap in real time to prevent the sample from either overflowing the plates (i.e., by increasing the gap as normal force increases) or shrinking (i.e., by decreasing the gap as normal force decreases). This is normally a feature of more expensive systems because it requires a normal-force sensor. Parallel-plate systems are necessary for this work; a cone-and-plate system cannot generally be used. In general terms, the instrument should be set to keep the normal force within 10% to 20% of its start value and also to allow changes in the gap of ~10% to 20%. Note that this feature works in addition to the gap temperature compensation present in most automatic gap-setting machines.

Data obtained over a wide temperature range for a polymeric material that is not thermosetting will reveal features around the glass transition (T_g; also known as the glass-to-rubber transition) such as peaks (G'' and tan[δ]) and onsets (places where a plateau gives way to a new linear region with a negative slope). These features and the temperatures at which they occur are often of great interest to the investigator. These are analyzed by the rheometer software in a systematic way to identify the temperature at which they occur.

COMMENTARY

The mathematics for deriving these parameters are fairly simple as long as the rules of linear viscoelasticity are obeyed. For this, the stress and strain used to do the calculation must be sufficiently small that they are in linear proportion. For example, if the applied stress amplitude is doubled, then the measured strain amplitude should also double, resulting in a relatively constant value for G^*. For polymers, this region of behavior is very long, because the nature of polymers is to withstand relatively large-scale deformations. Dispersions and emulsions are less rugged, as each component is relatively small and naturally sits in its own energy minimum. Any displacement from this position is therefore likely to be unfavorable.

Although the magnitude of the stresses defining the LVE region of behavior is important, it is not intuitive to the average person. What does a stress of 20 Pa feel like? Strains are perhaps more accessible. If we were to consider extension as a mode of deformation, we could consider the rack of Middle Ages renown as a form of tension tester used to test the resolve of its victims. A strain of 0.1% to 1.0% from such a device might be uncomfortable, but is essentially survivable as our bodies compress by that much—1 in. (2.5 cm) in a 72-in. (183-cm) adult—just from walking around all day. In the same sense, most materials will accept such strains without irreversible damage (flow). Similarly, just as we would not care to contemplate being stretched by 6 in. (15 cm) in height, most samples will deform irreversibly at strains of ~10%. Polymers, which can withstand up to 100% strain on occasion, are the exception. The presence of other materials (filler) will reduce this propensity.

All samples have to be loaded into the rheometer for testing, and this process will deform the sample in some way. For simple liquids (e.g., oils, sugar solutions), this is not a problem because they will recover instantly. In contrast, structured materials such as gels or pastes will be damaged. Changes in structure may result from molecular rearrangements or other actions such as drying through solvent loss. Some samples may recover (e.g., if they are thixotropic), whereas others may not (e.g., if they are gels and cross-links are broken). Whatever the sample, the loading process is traumatic, and thus the first step is to see if the sample is at equilibrium after being loaded (procedure 1). If it is still changing with time, then further experiments must wait until an equilibrium level of structure is reached. Structure is most conveniently defined as the magnitude of G', because this property is the most susceptible to damage during shear. Thus, measuring the changes in G' as a function of time (time sweep) will allow for accurate assessment of sample stability.

The torque sweep (or strain amplitude sweep in controlled-rate instruments) is the logical and necessary next step in assessing any material. Using increasing amplitudes, the sample is deformed in a reversible fashion (i.e., no net flow occurs), and the dynamic test is thus a sensitive mechanical real-time probe. This is especially useful if a sample is changing because of temperature effects—i.e., it is melting or going through a T_g. Alternatively, an isothermal change such as gelation or curing can be accurately tracked without the test itself interfering with the change of state. Thus, a time-sweep test or a temperature-sweep test is likely to be the method of choice for materials undergoing such physicochemical changes. Such

tests are a logical conclusion to the characterization tests, time sweep, torque or amplitude sweep, and frequency sweep.

A high priority for measurement is to define the LVE region, where the sample obeys the mathematical rules for the calculation of the various parameters. Data obtained outside of this region are qualitative only. The type of test used here is an amplitude sweep. The stress amplitude or the strain amplitude are varied at a constant frequency, and the region of linearity is clearly seen in a plot of G' (the most sensitive index of structure) versus the stress or strain. Increasing the stress or strain allows for the limit of viscoelastic behavior to be seen at a given frequency. Other methods rely on examining the sine waves themselves, as nonlinear data often result in visually deformed or asymmetric waveforms. An analysis of the third harmonic of the output is also insightful.

It is important to define the LVE region at all frequencies and temperatures of interest in subsequent tests. The length of the LVE region is frequency dependent; as the frequency decreases, the LVE region will shorten (narrow) as a function of stress and widen as a function of strain. The final aim of these preparative tests is often to examine the sample at different frequencies, performing a kind of mechanical spectroscopy upon it, and the data must remain within the linear response region at all times. It is important to remember that the response of a sample is a strong function of time. Elasticity can manifest itself at very short time intervals, but viscosity by definition is a time-consuming response. Thus, a test that scans frequencies will favor an elastic response at short times (high frequency) and a viscous response at long times (low frequency).

It is important to remember that all materials have a characteristic response time, varying from picoseconds for simple liquids like water to years for more traditional solids. If a sample appears to be a mobile liquid as it is disturbed in its container, then it will have a characteristic time of well under a second. If a sample appears to be an immobile solid, then it will have a characteristic time of several minutes or hours. Increasing the temperature of the sample will speed up molecular motion and thus decrease (shorten) the characteristic response time. Cooling the sample will have the opposite effect. If heating the sample is not an option, the only recourse is a long experiment!

Another aspect of measurement is the perception of the sample response. The simple polymer polydimethylsiloxane (PDMS), which is used to make Silly Putty, is a material that is an excellent model of viscoelastic behavior. Its characteristic response time does not vary at a constant temperature, although its apparent properties certainly do. When the container is opened, Silly Putty is seen to have found its own level just like a liquid, albeit a very viscous one. The moment it is subjected to a short impact, however, it bounces like an elastic solid. The difference is the amount of time allowed for the test; a long test is likely to reveal some liquid-like character, whereas a short test is likely to reveal only solid-like properties.

When taken to extremes, one can imagine even traditional liquids seeming to perform like a solid if the experiment is short enough. Consider the following thought experiment. From a boat, it is a pleasant experience to jump into a body of water. Imagine the same test repeated from an aircraft at high altitude without the benefit of a parachute. The water will not change but your perception of it most definitely will! Similarly, a sharp tug on an adhesive bandage is well known as the method of choice for its removal if a few body hairs are to be spared. The short time prevents the adhesive from responding like a liquid and wetting the skin or hairs as effectively. Otherwise, an extremely slow pull would allow the adhesive to flow away from the skin and hairs. Materials are tacky to the touch if they can flow into the crevices of the skin in a reasonably short time frame, but cooling the material inevitably makes it a little easier to handle. That is why pastry chefs recommend handling dough at low temperatures and with a protective coating of flour.

Samples are mechanically brittle at sufficiently low temperatures, normally below the T_g. They also are seen to be brittle at very short times. Thus, a short sharp impact can shatter a material at room temperature if it is naturally below its T_g or if the material has been frozen. Materials that are rubbery at room temperature can be sufficiently brittle at low temperatures to fail catastrophically. In the space shuttle Challenger, an explosion was caused by uncombusted fuel escaping when a rubber O-ring was rendered brittle by low-temperature weather. The key in all of these examples is the speed of molecular motion. For energy to be dissipated, a stress can cause a local increase in molecular motion. If that route is denied by time constraints (e.g., a fast shock) or temperature control (molecular immobility), then a crack is the only way energy can leak out. Cold toffee

is easily broken into bite sized pieces but will flex and flow into messy strings when left out to soften.

A frequency sweep can reveal these behavior patterns if it is collected over a sufficiently wide range. Low-frequency data are not usually difficult to obtain (provided that time is not a problem), but there are mechanical limitations to obtaining high-frequency data. At frequencies >10 Hz, it becomes impossible to correct for the inertia of the moving parts in the rheometer.

Other dynamic tests are more pragmatic in application, as they form a means of quantitatively monitoring the viscoelasticity of a material as it changes in real time or as a function of temperature. This means that melting, crystallization, gelation, and curing can all be followed without the test itself affecting the results.

Finally, oscillation data as a function of angular frequency can be used to extend the operating range of a flow test in unfilled polymer melts or solutions. These materials are often so elastic as to tear themselves out of the geometry gap during steady shear, even at relatively low shear rates (~1 Hz). An oscillation test can generate a value for complex viscosity (η^*) at a particular angular frequency that corresponds exactly to the value for η at the same shear rate. This empirical rule is known as the Cox-Merz rule and generally will not work for dispersions, emulsions, or filled polymer systems. Oscillation data can easily be generated at ~100 rad/sec, thereby extending the apparent range of the combined data by two orders of magnitude.

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The above references provide an excellent general reading list for more information on complex fluids.

Contributed by Peter Whittingstall
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Measurement of Gel Rheology: Dynamic Tests

BASIC PROTOCOL

This unit describes dynamic methods for monitoring the gelation process of protein dispersions and for determining rheological properties of the final gels. In a dynamic test, a test material is subjected to a controlled oscillation, the frequency of which determines the rate of deformation of the sample. The magnitude of deformation is kept very small to prevent destruction of gel structures during measurement. Two basic parameters reflecting elastic (G'; storage modulus) and viscous (G''; loss modulus) components of rheological properties are obtained at various frequencies. This frequency range will depend on the sample type and the purpose of the study (see Critical Parameters and Troubleshooting). The measuring instruments are operated by computers and the experimental procedure is simple. *UNIT H3.1* discusses much of the theory behind dynamic rheometer tests.

Materials

Sample protein solution or gel
Chemical substance to induce gelation, if needed
Immiscible reagent for preventing solvent evaporation (e.g., mineral or silicone oil), if needed

Dynamic rotational rheometer with appropriate test fixture
Computer with software package to control rheometer

1. Attach an appropriate test fixture to a dynamic rotational rheometer and connect rheometer inline with a computer.

   The appropriate text fixture type (or geometry) and size depend on the substance that is being analyzed. UNIT H1.2 includes a discussion on choosing a geometry.

   The amount of sample needed will depend on the rheometer and test fixture that are used. Ideally, there should not be excess sample (e.g., below or above the inner cylinder or outside the upper plate). Usually, sample below or above the inner cylinder does not contribute significantly to the results because of its smaller contact area compared with that of the wall of the cylinder. For steady rotational viscometry, it is often critical to cut the sample outside the upper plate. Thus it is also recommended to do so in dynamic tests. Changes in sample volume that often occur during gelation must also be considered.

2a. *For a gel induced by a chemical substance:* Mix a sample protein solution with a chemical substance to induce gelation, place the mixture in the test fixture, and cover it with an immiscible reagent.

   The chemical substance required to induce gelation, as well as the concentrations of chemical and protein, will need to be determined empirically.

2b. *For a gel induced by heating:* Place a sample protein solution in the fixture, cover it with an immiscible reagent, and use the computer to apply the appropriate temperature to the fixture for an appropriate length of time.

   The time and temperature of the incubation, as well as the protein concentration, will need to be determined empirically. Depednding on the purpose of the study, a temperature ramp or constant temperature can be used here and in steps 3 to 5.

2c. *For a preformed gel:* Place a sample gel in the fixture and cover it with an immiscible reagent, if necessary. Continue with step 4.

3. Set the computer to display G' and G''. Monitor G' and G'' during gel formation at, for example, 1 Hz and a maximum strain of 0.01.

   For a given gel, preliminary tests are required to determine an appropriate frequency and strain.

Contributed by Shinya Ikeda and E. Allen Foegeding
Current Protocols in Food Analytical Chemistry (2003) H3.2.1-H3.2.9

4. Measure the frequency dependence of G' and G'' after completion of the gelation.

   For a further discussion of a frequency sweep test, see UNIT H3.1. The range of frequencies used here and in step 5 will depend on both the sample and the rheometer type.

5. Measure the strain dependence of G' and G''.

COMMENTARY

Background Information

Recent advances in dynamic rotational rheometers are of growing importance in food analyses for several reasons. (1) The measurement minimizes the destruction of the material. (2) The time required for a measurement is reasonably short in comparison with chemical or physical changes in the material. (3) The viscoelasticity of gels is characterized by determining G' and G'' in the linear viscoelastic (LVE) region; no other method gives dynamic moduli values.

Protein gelation is induced by many factors such as temperature, pH, or additives. Protein molecules in a medium become reactive under appropriate conditions and, at a certain probability, will stick to one another upon collision. The resultant aggregate diffuses and forms larger aggregates by linking to other molecules or aggregates. If the protein concentration is sufficiently high, a network structure percolating in the entire system is eventually formed. This overall process is called gelation. Because protein gels generally show combinations of ideal rheological behavior (i.e., the behavior of both an ideal elastic solid and an ideal viscous fluid), protein gels are considered viscoelastic materials.

The relative magnitudes of elasticity and viscosity for a viscoelastic material depend on the scale of the observation time. If the rate of a deformation is very slow, a material may behave more like a viscous fluid. The faster the deformation occurs, the more elastic the material appears. Therefore, in the measurement of viscoelasticity, the rate of deformation should be chosen based on a practical situation of interest (e.g., the rate of processing or mastica-

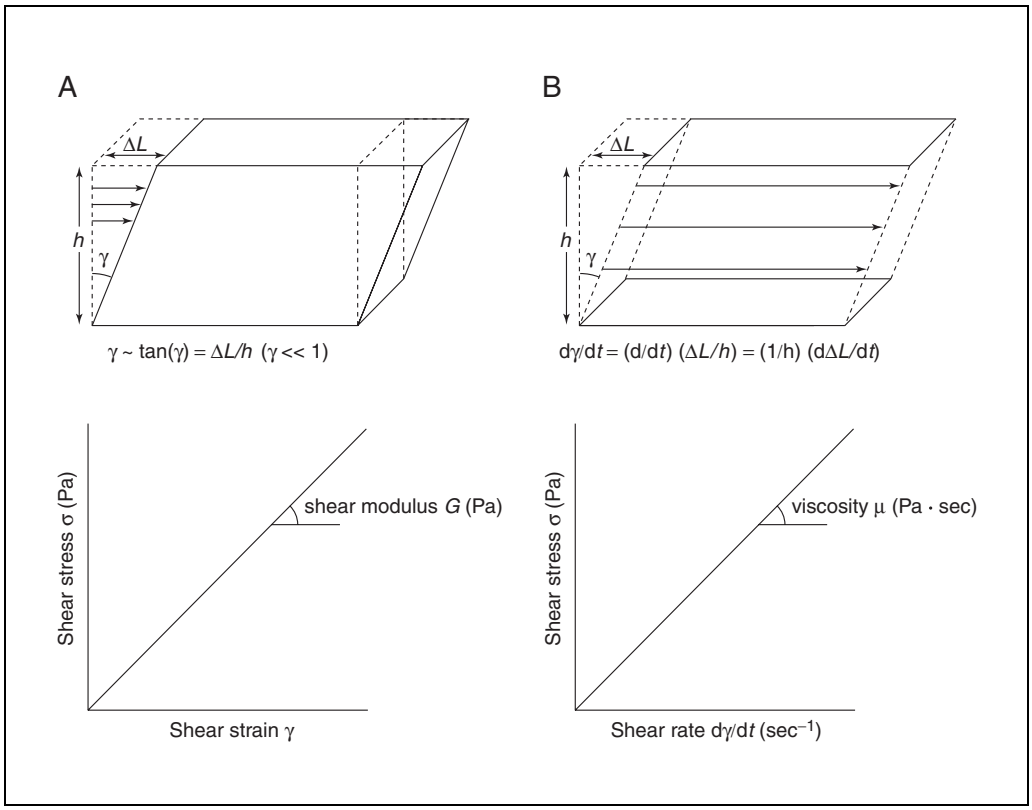

Figure H3.2.1 Deformation pattern of a substance in response to shear. (**A**) An ideal elastic solid subjected to shear. (**B**) An ideal viscous fluid subjected to shear. *h*, height; Δ*L*, displacement in length.

tion) to obtain a meaningful result. From another point of view, a material can be characterized by determining its mechanical response over a wide range of deformation rates, which is also within the scope of a dynamic test.

An elastic solid has a definite shape. When an external force is applied, the elastic solid instantaneously changes its shape, but it will return instantaneously to its original shape after removal of the force. For ideal elastic solids, Hooke's Law implies that the shear stress (σ; force per area) is directly proportional to the shear strain (γ; Figure H3.2.1A):

$$\sigma = G\gamma$$

Equation H3.2.1

where the proportional constant G (Pa) is the shear modulus, a measure of the elastically stored energy (see also *UNIT H3.1*). In contrast, a viscous fluid has no definite shape and flows upon application of an external force. Applying a shear stress to an ideal viscous fluid (Newtonian fluid) results in a homogenous layer flow (Figure H3.2.1B). A material can be characterized by a parameter reflecting an internal resistance to flow. For a Newtonian fluid, the flow rate, expressed as shear rate ($d\gamma/dt$), is directly related to shear stress:

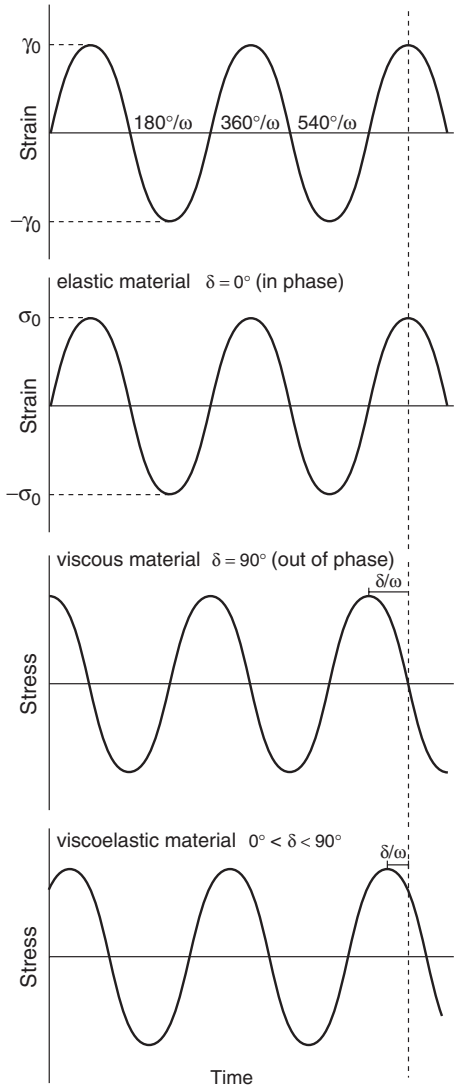

Figure H3.2.2 Responses of an ideal elastic, viscous, and viscoelastic material to a sinusoidal deformation. δ, phase angle; γ, shear strain; ω, angular frequency; σ, shear stress.

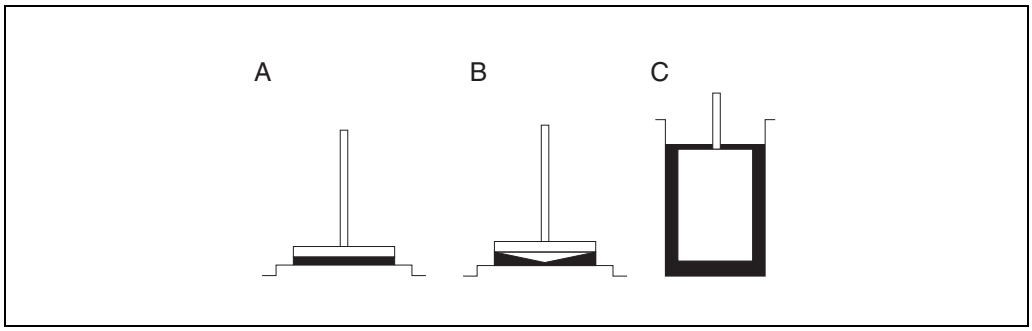

Figure H3.2.3 Test fixture geometries. (**A**) Parallel plates. (**B**) Cone and plate. (**C**) Concentric cylinders. The black regions correspond to sample material.

$$\sigma = \mu \frac{d\gamma}{dt}$$

Equation H3.2.2

where the proportional constant μ (Pa·sec) is Newtonian viscosity.

A viscoelastic material is represented by a combination of elastic and viscous bodies. If a controlled sinusoidal small strain (γ) is applied to a viscoelastic material as follows:

$$\gamma = \gamma_0 \sin(\omega t)$$

Equation H3.2.3

then the stress response (σ) is:

$$\sigma = \sigma_0 \sin(\omega t + \delta)$$

Equation H3.2.4

where γ_0 is the strain amplitude (maximum strain), σ_0 (Pa) is the stress amplitude (maximum stress), ω is the angular frequency (which is equal to $2\pi f$, where f is the frequency), and δ is the phase angle, reflecting the phase shift between γ and σ due to viscoelasticity. Equation H3.2.4 can also be expressed as the sum of in-phase and 90°-out-of-phase components with the strain:

$$\sigma = G'\gamma_0 \sin(\omega t) + G''\gamma_0 \cos(\omega t)$$

Equation H3.2.5

where G' (Pa) is the storage modulus and G'' (Pa) is the loss modulus. When comparing Equation H3.2.5 with Equations H3.2.1 and H3.2.2, one notices that the first and second terms of Equation H3.2.5 are the same form as Equations H3.2.1 and H3.2.2, respectively. That is, the term $G'\gamma_0\sin(\omega t)$ is in the form of a constant (G') multiplied by strain ($\gamma_0\sin(\omega t)$), which is the same as Equation H3.2.1. The term $G''\gamma_0\cos(\omega t)$ is in the form of a constant (G''/ω) multiplied by shear rate ($\omega\gamma_0\cos(\omega t)$), which is the same as Equation H3.2.2. Note that the

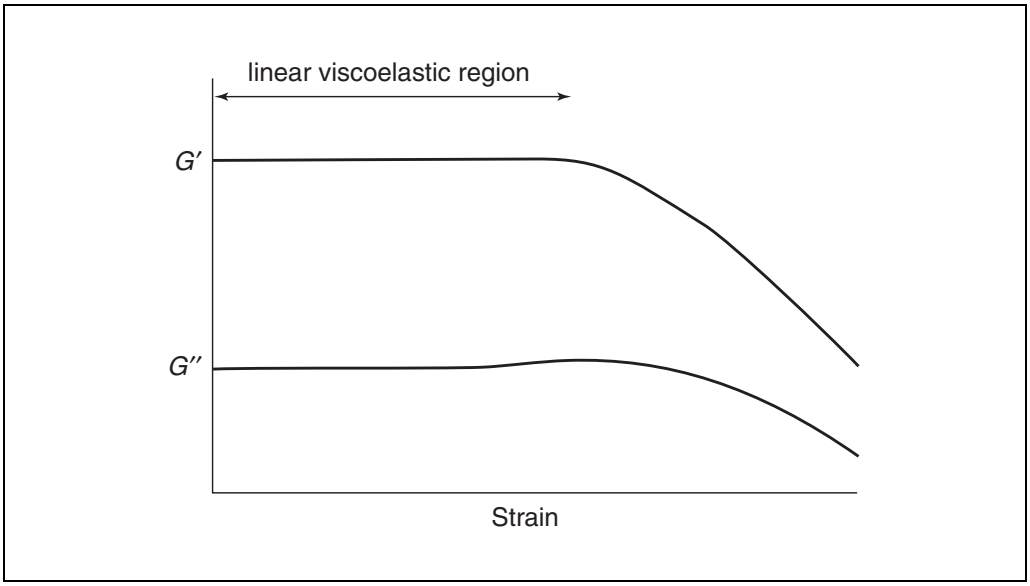

Figure H3.2.4 Linear viscoelastic region as determined by the strain dependence of G' (storage modulus) and G'' (loss modulus).

shear rate is given by differntiating Equation H3.2.3 with respect to time, $d\gamma/dt = \omega\gamma_0\cos(\omega t)$. Therefore, the storage modulus G' reflects the elastic component of viscoelasticity, namely, a measure of the elastically stored and recovered energy per cycle of deformation, and the loss modulus G'' reflects the viscous component, namely, a measure of the energy dissipated as heat. For an ideal elastic solid, the entire applied energy is stored (i.e., $G'' = 0$), and thus the strain and the stress are in phase. For an ideal viscous fluid, all the applied energy dissipates as heat (i.e., $G' = 0$), and thus the strain and the stress are 90° out of phase. These relationships are schematically illustrated in Figure H3.2.2.

By comparing Equations H3.2.4 and H3.2.5, the following equations are obtained.

$$G' = \frac{\sigma_0}{\gamma_0}\cos(\delta)$$

Equation H3.2.6

$$G'' = \frac{\sigma_0}{\gamma_0}\sin(\delta)$$

Equation H3.2.7

$$\frac{G''}{G'} = \tan(\delta)$$

Equation H3.2.8

The term $\tan(\delta)$ is referred to as the loss tangent, a measure of the relative magnitude of the viscous to the elastic component, or the relative magnitude of lost to stored energy per cycle deformation. Predominately elastic (solid-like) material has a $\tan(\delta) < 1$ ($G' > G''$), whereas a fluid-like material has a $\tan(\delta) > 1$ ($G' < G''$). Additional parameters can be defined, such as the complex modulus G^* ($G' + iG''$; where i is the imaginary unit).

$$\sigma = G^*\gamma$$

Equation H3.2.9

$$|G^*| = \sqrt{G'^2 + G''^2}$$

Equation H3.2.10

Hooke's Law, which states that a proportional relationship exists between stress and strain, usually holds for a viscoelastic material at a small strain. This phenomenon is called linear viscoelasticity (LVE). Within the LVE region, the viscoelastic parameters G' and G'' remain constant when the amplitude of the applied deformation is changed. Consequently, parameters measured within the LVE region are considered material characteristics at the observation time (frequency).

Various rheological instruments have been developed. Some instruments are designed for a specific purpose and give only instrument-specific parameters. A gel rheology test using this type of instrument is called an empirical test, which is of value if a correlation with a property of interest is found. A fundamental test has the advantage over an empirical test in determining true (i.e., material-characteristic and instrument-independent) physical properties. Some fundamental rheological tests apply a large deformation up to the point at which the material fractures, and they correlate well with

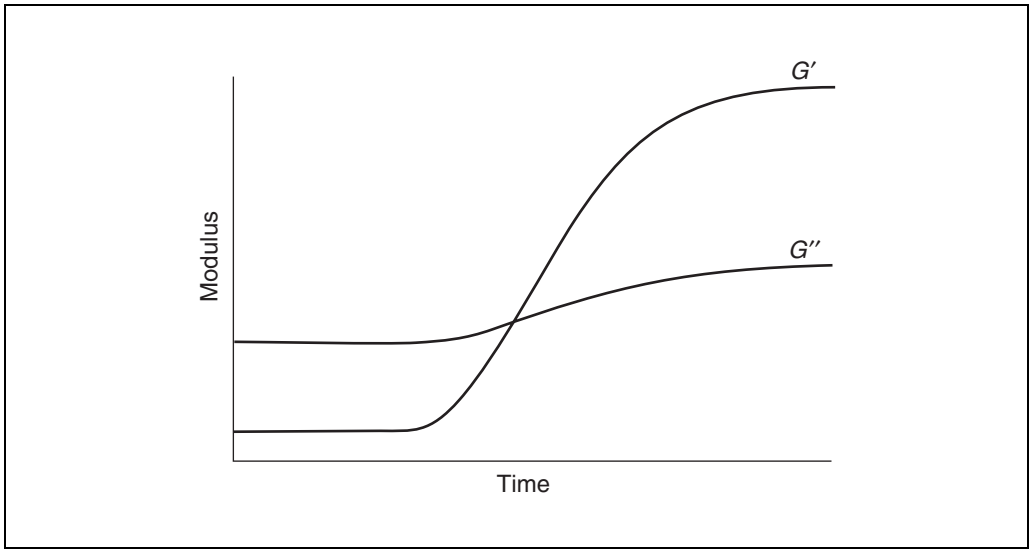

Figure H3.2.5 Development of moduli during gelation. G', storage modulus; G'', loss modulus.

results of sensory texture analyses (Montejano et al., 1985). A large deformation, however, can push the material beyond its LVE region, at which point viscoelasticity becomes a function of the magnitude of the deformation as well as of time. If this is the case, a mathematical or quantitative interpretation of the results is very complex, if it is even possible. Therefore, a fundamental test applying a small deformation is more suitable for analyzing the general rheological properties of food gels.

Critical Parameters and Troubleshooting

Most rheometers that are commercially available for a dynamic test are designed so that a controlled strain or stress is applied to the test material (Bohlin et al., 1984). A sample is placed between the two parts of a test fixture (i.e., two plates or a cone and plate). For most strain-controlled instruments, the rotationally controlled piece transmits an input to the sample and the response is detected by the stationary piece. The common types of test fixtures are the cone and plate, parallel plates, and concentric cylinder (Shoemaker et al., 1987; Figure H3.2.3). Cone-and-plate and parallel-plate fixtures are easy to set up and facilitate the calculation of dynamic moduli, whereas the concentric cylinder fixture is preferred for preventing water loss because the sample surface can easily be covered with immiscible fluids.

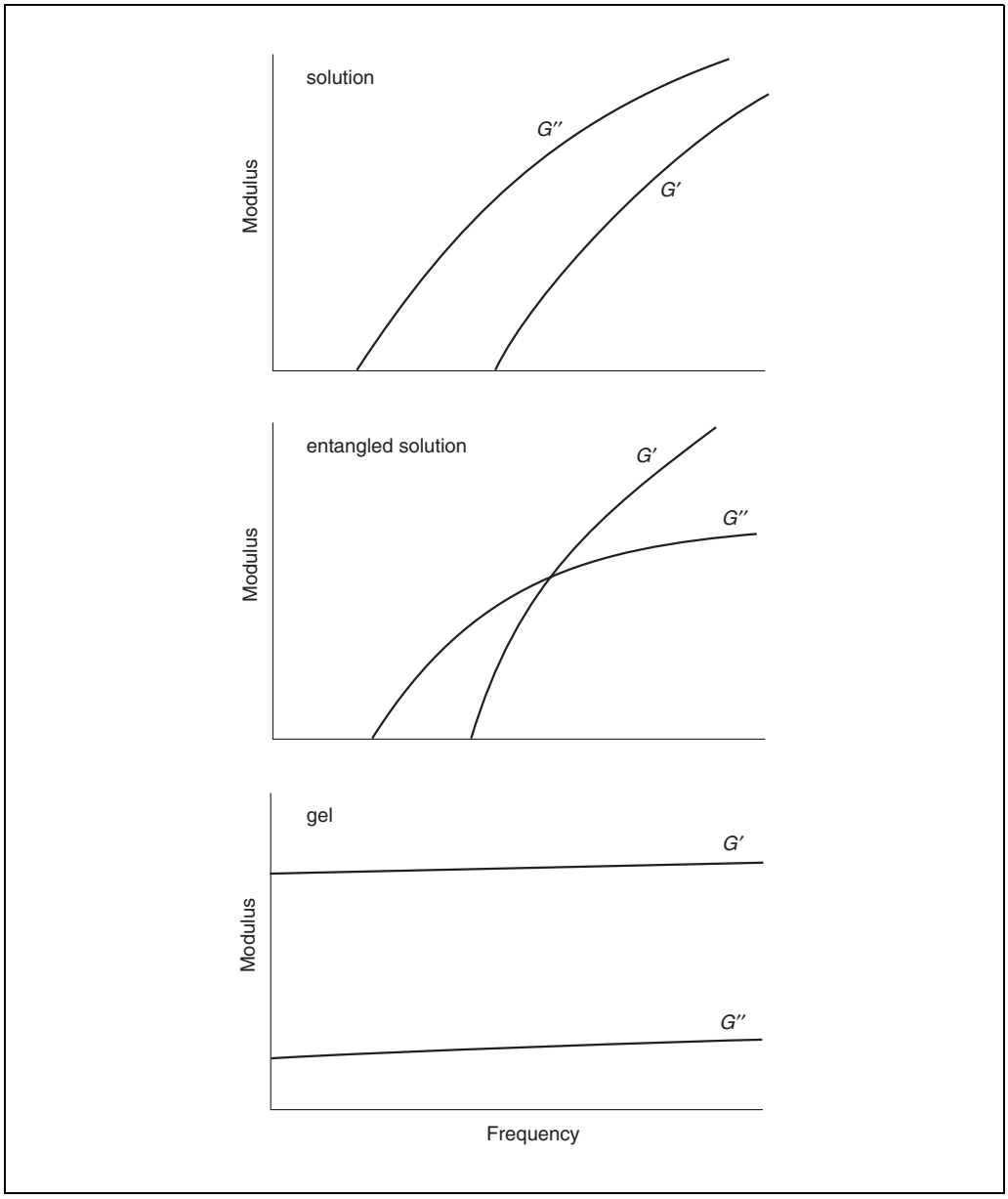

Figure H3.2.6 Three types of materials that are rheologically distinguishable based on a frequency sweep test. *G′*, storage modulus; *G″*, loss modulus.

The size of the fixture is chosen based on the testing material and the sensitivity of the instrument. (Usually, various sizes and types of fixtures are available for an instrument.) Generally, responding torque becomes larger as the diameter of the fixture is increased or as the gap between the fixture surfaces decreases.

Before starting a gelation process, the operator needs to select the magnitude and frequency of the applied strain or stress. Theoretically, smaller applied deformations and lower frequencies are less likely to damage the gel network structure. A lower limit of deformation exists, however, to gain a good signal-to-noise ratio. At the same time, the magnitude of input should be chosen to ensure LVE as follows. The LVE region of the testing sample is established by running stress or strain sweeps on the material. In each case, the magnitude of the input is increased while moduli are determined (Figure H3.2.4). The LVE region is observed at a low deformation region where the moduli remain constant. As the magnitude of the input is increased, the moduli will suddenly start to decrease or increase at a certain strain or stress value, which is the limit of LVE. If various frequencies are used in the test, strain or stress sweep measurements should be done at least at the upper and lower extremes of the frequency range to ensure linearity at all frequencies. The strain-controlled measurement is recommended for testing materials that show a drastic change in rheological properties during the measurement (e.g., from sol to gel), because a stress necessary for measuring a strong gel would cause a substantial strain when applied to a weaker gel, which may exceed the LVE region.

In the case of heat-induced gelation, a step increase in temperature may not be available for some instruments. Alternatively, the temperature is raised at a fixed rate while the rheological parameters are monitored. Various kinetic phenomena such as denaturation and aggregation of proteins take place as the temperature increases, and thus the results obtained during heating cannot be considered absolute, as they depend at least on the heating rate. Attention should be paid to the heating rate or the size of the sample when comparing the results with other analytical measurements such as differential scanning calorimetry (DSC).

Anticipated Results

Figure H3.2.5 schematically shows changes in G' and G'' when a protein solution is placed under a constant high temperature. Prior to gelation, the material shows a typical fluid-like behavior ($G' < G''$). If the size of protein aggregates becomes large enough, G' increases rapidly, and after some time, a cross-over point ($G' = G''$) is observed. This point and the corresponding time are often referred to as the gel point (gelation point) and the gel time (gelation time), respectively (Clark and Ross-Murphy, 1987; Djabourov, 1988; Clark, 1992). As gelation progresses, G' becomes dominant, showing characteristics of solids ($G' > G''$). In some test systems, a cross-over of G' and G'' may not be observed. As G' is initially almost zero

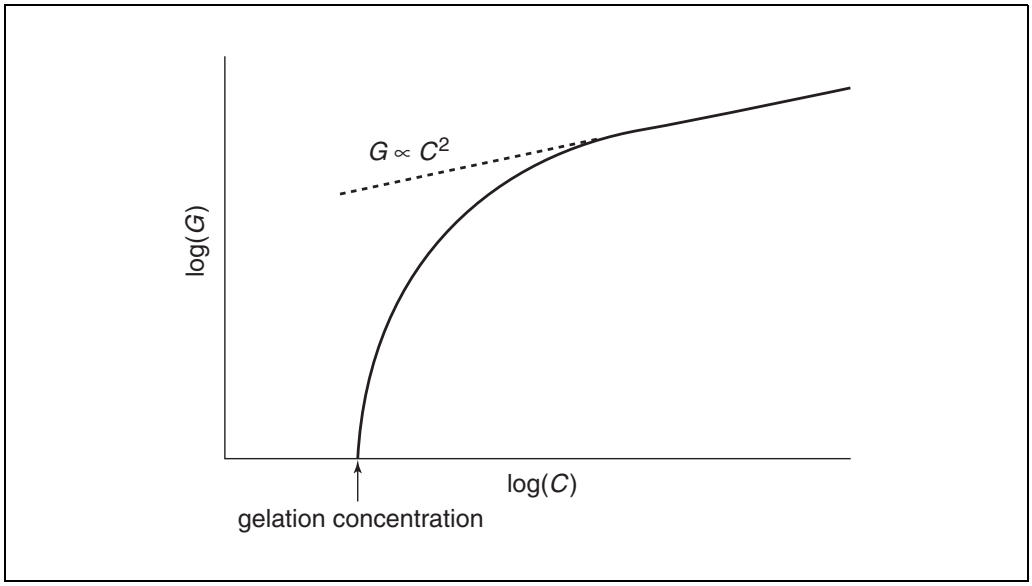

Figure H3.2.7 Concentration dependence of gel modulus (G). G can be either the storage modulus, G', or the complex modulus, G^*, as both usually coincide within expermiental error. C, concentration.

because of the fluid-like nature of the material, the gelation point can be defined as the point where G' begins to increase rapidly. This point may be determined by an extrapolation depending on the sensitivity of the measuring instrument. It should be noted that the gel point determined by this procedure usually depends on the measuring frequency. Theoretical aspects of determination of the gel point have been considered in the literature (Winter, 1987; Scanlan and Winter, 1991). Comprehensive reviews regarding theoretical treatments of the gelation process of biopolymers are also available (Clark and Ross-Murphy, 1987; Ziegler and Foegeding, 1990). Although the fundamental theories of gelation were developed for describing the gelation process of synthetic polymers via linking by covalent bonds (Stockmeyer, 1943; Flory, 1953), modern progress in physics, such as advances in percolation theories, has allowed their application to the analysis of a sol-gel transition in protein gels (de Gennes, 1979).

A strain or stress sweep is used to establish the LVE region (Figure H3.2.4). The LVE region is a characteristic of a material. While the strain value at the limit of LVE rarely exceeds 0.1 for colloidal gels, a larger LVE region with a strain of up to 1 or more is usually observed for biopolymer gels (Clark and Ross-Murphy, 1987).

Although it is almost impossible for a protein gel to reach an equilibrium state (G' continues to increase gradually for hours or days), G' becomes approximately constant a few hours after gelation time (Figure H3.2.5). A gel that has experienced an aging process can be called a cured gel, and a characterization of a cured gel is also in the scope of a dynamic test. Although available frequencies are usually limited to a range of 0.001 to 10 Hz, frequency sweep measurements within the LVE region reveal the type of material (Djabourov, 1988; Scanlan and Winter, 1991; Ross-Murphy, 1994).

Typical patterns of G' and G'' produced from frequency sweep experiments are classified into three categories (Figure H3.2.6). A dilute solution of macromolecules shows $G' < G''$ at most frequencies. With increasing concentration, interactions among molecules become pronounced in what is called an entangled or semi-dilute solution. An entangled solution has a distinguishing feature of a cross-over point where $G' = G''$ or $\tan(\delta) = 1$. The material is fluid like at a frequency below the cross-over point, but solid like at a frequency above the cross-over point. The frequency at the cross-over point is thus also referred to as the relaxation frequency. If a gel is formed, G' is predominant ($G' > G''$) and both G' and G'' are relatively independent of frequency. If ideal cross-links are formed by permanent covalent bonding, the moduli are completely independent of frequency. A protein gel, however, usually shows a slight frequency dependence and is called a physical gel. When $\log(G')$ is plotted against $\log(f)$, the slope is slightly greater than zero and is typically less than 0.1. More elastic gels have lower slope values, whereas more viscous gels have higher slope values.

There have been attempts to widen the frequency scale based on a procedure called time-temperature superposition (TTS; Tokita et al., 1983; Dea et al., 1984; *UNIT H3.1*). Because this procedure assumes that only the number and the strength of cross-links are dependent on temperature, it must be determined whether the material undergoes physical or chemical changes following temperature changes. It may be possible to widen the frequency range using a few different methods or instruments based on different principles (Nishinari, 1976), although a substantial change in the shape of the spectrum may not be expected (te Nijenhuis, 1981).

The concentration (C) dependence of G' has generated much interest not only from a practical point of view but also from a scientific standpoint. When $\log(G')$ is plotted against $\log(C)$, a power law relationship is often observed. The slope of the power law is dependent on the concentration range: the slope is large (5 to 7) when the concentration is close to the critical gelation concentration, becomes smaller with increasing concentrations, and then converges to a value close to 2 (Figure H3.2.7). Regarding the relationship between G' and C, various models (Hermans, 1965; Oakenful, 1984; Clark and Ross-Murphy, 1987), including the recent introduction of fractal geometry (Bremer et al., 1990; Shih et al., 1990), have been developed.

Time Considerations

Because the duration for one measurement is very short (e.g., with a 1-Hz input, a cycle is completed in 1 sec), a dynamic test is suitable for gaining information in a short time frame or for monitoring time-dependent changes in gel network properties. When monitoring the gelation process at a fixed frequency, it usually takes a few hours for G' to become approximately constant. The constancy can be judged by a constant value of G' at a fixed frequency during a subsequent frequency or strain sweep test, which usually takes several minutes.

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Creep and Stress Relaxation: Step-Change Experiments

BASIC PROTOCOL

Rheometers can impose a rapid step change using their drive systems and measure the response of the sample as a function of time. These output signals can be analyzed to extract viscoelastic information, including relaxation times. Initially, when elapsed time is small, the response is analogous to high-frequency oscillation data; however, as the elapsed time increases and the material reaches steady state, the response correlates with very low-frequency data. If steady state is reached, the data can sometimes be mathematically converted to ultra-low-frequency information (*UNIT H3.1*). Thus, these techniques are often a shortcut used to obtain so-called terminal zone data (data that reflect the longest relaxation times that can be measured). These types of test are easy to assess visually because the shape of the curve is directly related to the behavior of the sample. The more linear the output, the more it is dominated by liquid properties. The more curved the output, the more it is dominated by solid-like behavior.

A controlled-stress instrument imposes a step stress, and such experiments are referred to as creep tests (Fig. H3.3.1). Typically there are one or two steps in a creep test: the imposition of a stress (retardation step) and its removal (relaxation or recovery or recoil step). The output signal is displacement, often displayed as strain, % strain, or compliance, *J* (strain/stress). The latter signal is displayed "as is" in the creep step, but in the recoil phase is calculated by subtraction from the previous step (zero stress would cause a nonsensical quotient), and is thus more properly called the recoverable compliance. When a creep step is analyzed, the latter linear part of the curve is associated with a viscous response, so a viscosity at a given stress (applied) and shear rate (measured) is generated. Creep can be considered as the precursor to flow, since experiments can be performed in either the linear viscoelastic region of a sample or the nonlinear region where irreversible deformation (i.e., flow) begins to dominate. Thus, creep tests can be used to probe the zero shear plateau or maximum apparent viscosity of a material as well as the onset of shear thinning. If this onset is sharply defined, the material is said to have a "yield stress," and the creep test is perhaps the most reliable way of evaluating this behavior.

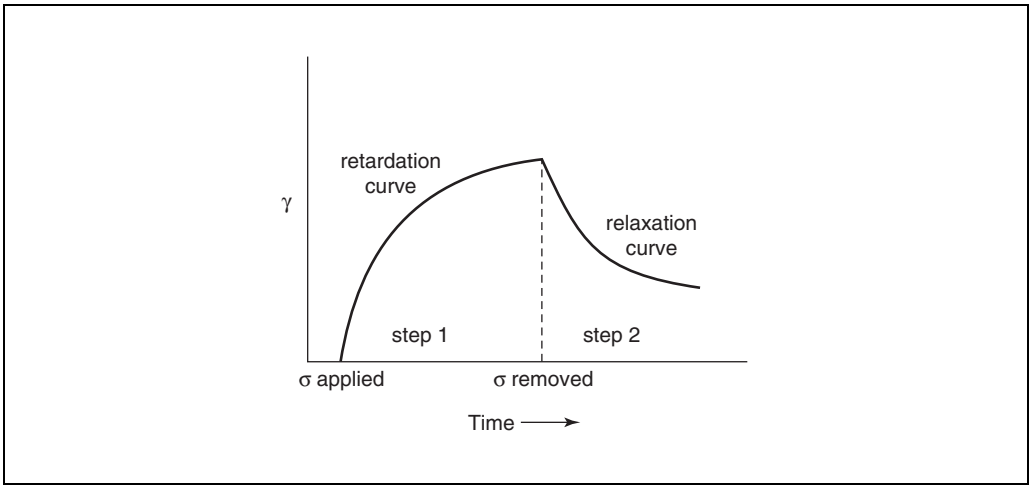

Figure H3.3.1 A creep experiment where a small constant stress (σ) is applied to a food sample (step 1) for a period of time. Afterwards the applied stress is removed (step 2). The degree of deformation (strain, γ) is measured during the experiment, and a typical response is shown.

Contributed by Peter Whittingstall
Current Protocols in Food Analytical Chemistry (2003) H3.3.1-H3.3.8

Controlled-strain instruments do not perform creep tests well because their natural mode of operation is the rapid imposition of position changes or speed changes. The equivalent controlled-strain step-change test is called a stress relaxation test. In this test, the drive system rapidly changes the position of the rotor through a certain angular displacement (strain). The output signal is torque, often displayed as stress or modulus, *G* (stress/strain). The detector picks up the sample's response to this change in position as a sharp rise in stress (torque). For a liquid sample this stress will decay rapidly as energy is dissipated within the fluid. The response shape will thus resemble a spike. A solid, on the other hand, can store energy, and so the rise in stress will be maintained as a plateau. Real materials are viscoelastic and thus give an exponential decay in stress. The exponential time constants are related to the viscoelastic parameters seen in the creep curves and have the same significance (Fig. H3.3.2).

The viscoelastic samples to be tested by this method may be in different forms. The simplest to work with is a soft or liquid-like viscoelastic material such as mayonnaise or other food emulsions. These are easy samples to work with terms of sample loading. More solid-like samples such as cheese or food gels are more difficult to load onto the instrument in a consistent matter. The degree of compression of soft samples should ideally be controlled using a normal force measure or force rebalance system. Slippage is also a concern and roughened plates or even adhesives may be needed if slip is an issue. As this protocol is a general one, it is assumed that the sample is already loaded on the rheometer and has achieved equilibrium in terms of temperature and viscoelastic structure (time-dependent behavior).

Materials

Sample (prepared or intact)

Rotational rheometer (*UNIT H1.1*; e.g., Bohlin Instruments, Chandler Engineering): controlled stress (for applied step shear stress) or controlled strain (for applied step shear strain) with appropriate software for rheometer control, data acquisition, and data analysis

Appropriate testing fixtures (Fig. H1.1.1): parallel plates are generally preferred for these tests

1. Load sample (*UNITS H3.1 & H3.2*), then select a stress (creep test) or strain (stress relaxation) for use in the test.

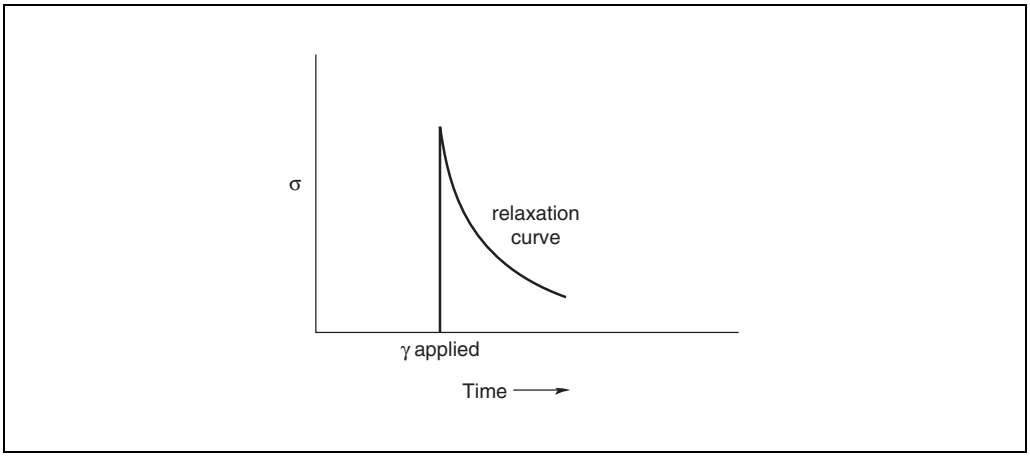

Figure H3.3.2 A stress relaxation experiment where a small constant strain (γ) is applied to a sample. After the strain is applied, the stress (σ) of the material is measured as a function of time.

The stress or strain selected should be in the linear viscoelastic region of the sample measured at a frequency of ~1 Hz or 10 radians/sec (1.6 Hz; UNIT H3.1; also see Background Information). Choose a value that is not close to the upper limit of the linear region, as a sample may leave the linear region of behavior during a prolonged step change test. Occasionally, a specific stress or strain value that the sample will face during use may be applied, where it is of interest to see if the sample recovers or deforms/flows irreversibly in response. If the linear viscolelastic region is not known, consider running an oscillation amplitude sweep using stress or strain as appropriate for the instrument. Sometimes this option is not available, so trial and error is also an alternative.

2. Apply several stresses or strains, starting low and working up. If possible, monitor the output signals of the transducer or position sensor while applying test input signals. When the signal is relatively noise free, repeat that step as a complete test.

   It is often advisable to load a new sample at this point.

   Most instruments have a mode that allows output monitoring.

   The critical phase of either a creep test or a stress relaxation test is in the first few seconds. It is here that the transducer can be overloaded or the optical encoder can give a noisy response. Most controlled-strain instruments will give an audible error signal if the transducer is overloaded. For controlled-stress instruments, the noise level is determined by the resolution of the optical encoder.

3. Once the step value is determined, apply it for at least 5 min, preferably 15 min or more.

   The sample must have reached steady state before cessation of the test or the application of a second step. Steady state in a creep test is seen as a constant slope in the strain curve. A constant slope in the stress curve may also be seen in a stress relaxation test, but often the signal is lost in the noise. A material that is liquid-like in real time will need a test period of ~5 to 10 min. A stress relaxation test is likely to be somewhat shorter than a creep test since the signal inevitably decays into the noise at some point. A creep test will last indefinitely but will probably reach steady state within an hour. For a material that is a solid in real time, all experiments should be longer as molecular motion is, by definition, slower. Viscoelastic materials will lie in between these extremes. Polymer melts can take 1 hr or more to respond in a creep test, but somewhat less time in a stress relaxation test.

   If the software offers an equilibrium tolerance option to stop the test prior to the allotted time, disable it. It is better to compare tests that have been run for the same amount of time.

4. *For creep tests:* Perform a recoil or recovery step using a segment of zero applied stress. Use a step duration that is at least as long as the retardation step.

   The recoil step is sometimes deliberately set to be longer than the retardation step in order to ensure complete recovery to steady state.

   Creep tests typically have a recoil step, unless they are of a multistep variety. For instance, some workers will use multiple retardation steps of increasing stress to visually determine the apparent yield stress or onset of flow. This is seen as a sharp biphasic response in the strain. The same yield behavior can be seen by plotting several creep curves on top of each other. When plotting compliance curves for the retardation step, the data will superimpose if the sample is in the linear region (stress is proportional to strain by definition), due to the normalizing effect of dividing the measured strain by the applied stress. As soon as the linear region is exceeded, the curves will no longer lie on top of each other.

   Stress relaxation tests need not have a second step, although some workers recommend a second step in the opposite direction. The Boltzmann superposition principle for polymers allows for multiple step-change tests of both types (stress or strain) as long as the linear limit of the polymer is not exceeded (Ferry, 1980).

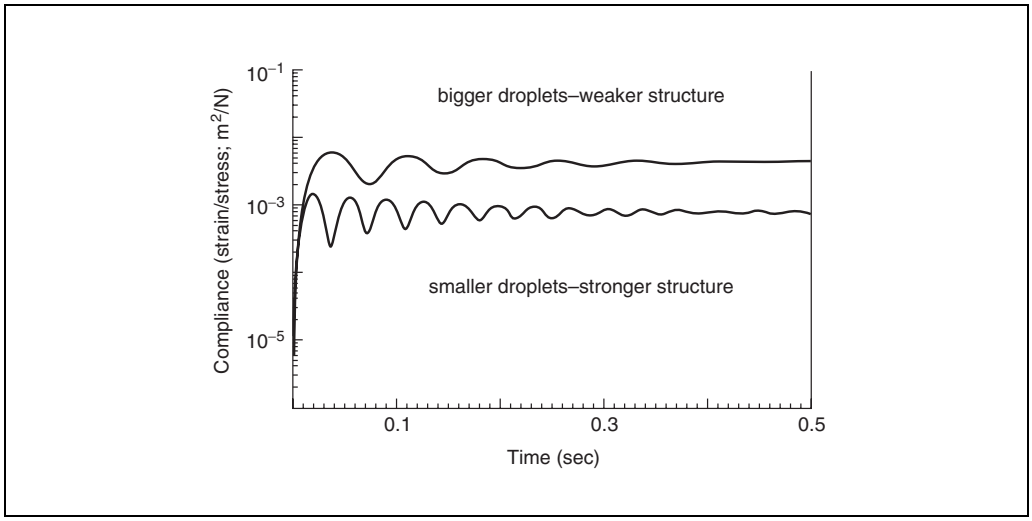

Figure H3.3.3 The immediate response to an imposed stress upon two emulsions produces two different responses with respect to size of the emulsion droplets.

5. Analyze data.

The data from step-change tests is modeled mathematically to fit one or more exponential time constants depending upon the curvature of the line. Each exponential time constant is related to a relaxation time in the sample response. Most rheometers have software that automatically fits the data to these models.

It is often necessary to ignore the first second of data from a step change test when using such models. The first few data points of a creep test often reflect a "ringing" in the sample if there is a gel-like structure present. An example is shown in Figure H3.3.3, where two emulsions are mixed at different speeds with the same formulation (in terms of, for example, stabilizers). The sample mixed at lower speed has larger droplets and therefore a lower surface area for the emulsifiers to work with. The difference is clearly seen in the pattern of damped oscillations. This damped oscillation is very useful because it can provide insight into the elastic properties of the sample, but it cannot be modeled successfully by the Burgers model. It must therefore be excluded.

The first second of a stress relaxation step can also show this type of ringing, but it is generally caused by the transducer itself. Thus, the first part of the data may be electronically filtered to remove the transducer ringing by setting a filter cutoff frequency of ~40% of the value for the resonant frequency of the transducer and geometry. Some rheometers allow for the measurement of transducer resonant frequency when measuring the geometry inertia.

Other mathematical transformations of the data allow for low-frequency oscillation data to be calculated from step change data, which is desirable since step change tests are generally quicker than low-frequency studies. This technique is outside the scope of these protocols, but interested readers are referred to Ferry (1980).

COMMENTARY

Background Information

Creep tests

The stresses used in a creep test are chosen in two ways. First, a value is chosen from oscillatory tests (specifically stress or strain amplitude sweeps at 1 Hz or 10 rad/sec; *UNIT H3.1*) to define the linear region. Using two to five different values, the sample is taken from a linear viscoelastic response to the onset of flow. This may require stresses to be chosen over an order of magnitude or more. Flow tests are then used to further characterize the sample.

The second method is to calculate a stress that is appropriate to a particular situation of interest. An example of this would be the stress acting on a drop of material due to its own weight as it rests on a support medium. The force of gravity tends to make the drop spread out into a film, while its surface tension tends

to resist this process. The resulting force can be converted to a stress by knowing the footprint (surface area) of the drop. In the confectionary industry, the ability to dispense shaped candy is important for product consistency. The creep test at the calculated stress will predict how well different batches of material will perform.

All creep curves can be used to derive a viscosity value. This value is obtained from the slope of the output once steady state has been reached, and the exercise should not be attempted if the output is still changing in terms of its slope. On occasion, the only way to be sure that further changes will not occur is to analyze a particular curve and then repeat the experiment for a slightly longer time and reanalyze it. If the same results are obtained, the sample has reached steady state. It typically takes between 5 min and 1 to 2 hr for a material to achieve steady state. More viscous samples will take more time than less viscous samples. At low stresses the values for the viscosity can be very large since the measured shear rate is often very small. If the data are analyzed in the linear viscoelastic region, it is a good method for deriving zero shear viscosity.

The normal method for analyzing creep curves is to use the Burgers model (Barnes, 2000; Figure H3.3.4), where a spring and a dashpot represent the very short-time and steady-state long-time behavior, respectively. In between these extremes are as many as four Kelvin-Voigt units comprising a spring and a dashpot linked in parallel with each other so that they experience the same strain. These viscoelastic elements each have a relaxation time (or more properly a retardation time) associated with them. A typical data fit will therefore list an initial compliance (or strain) associated with the single spring (J_0) and a steady-state or Newtonian viscosity (η) and shear rate associated with the single dashpot (Fig. H3.3.5). After that will be listed the compliance, viscosity, and relaxation time for as many Kelvin-Voigt units as are needed to fit the line. Finally, some kind of goodness of fit will be described (e.g., standard error). Remember that the viscosities from creep tests in the linear viscoelastic region will equate to the zero shear viscosity. Differences in zero shear viscosity and/or J_e^0 (recoverable compliance) are sometimes related to differences in molecular weight distribution (MWD) in polymers or particle/droplet size distribution in dispersions.

The steady-state viscosity is a pragmatic way of predicting certain key properties of a sample. If the stresses used in the creep experiment are well chosen they will reflect the stresses applied to the sample by the action of gravity. Thus, the viscosity under these circumstances will help predict the ability of the material to resist sagging on a vertical surface (coatings). Other uses include prediction of sedimentation velocity or creaming velocity in two-phase dispersions. The ability of a paint to level out and therefore remove brush marks by

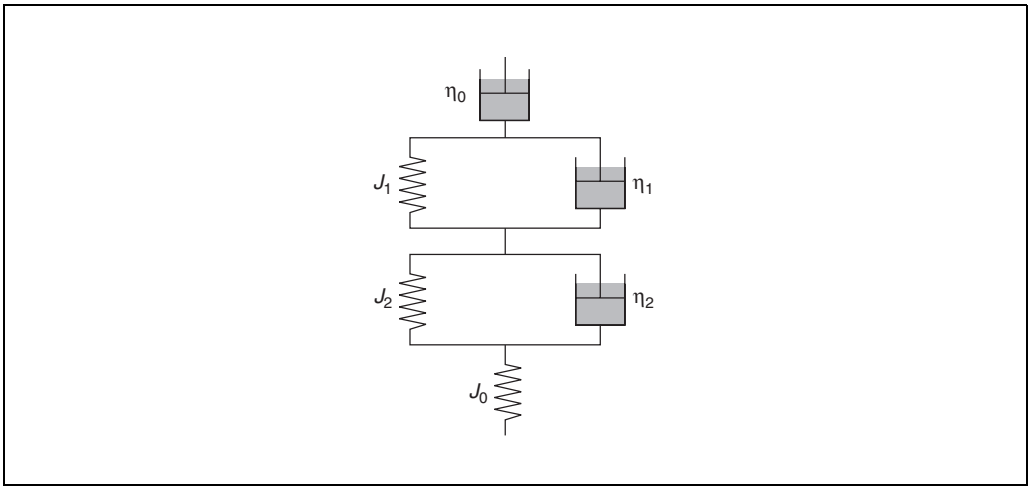

Figure H3.3.4 Mechanical models are often used to model the response of foods in creep or stress relaxation experiments. The models are combinations of elastic (spring) and viscous (dashpot) elements. The stiffness of each spring is represent by its compliance (J = strain/stress), and the viscosity of each dashpot is represent by a Newtonian viscosity (η). The form of the arrangement is often named after the person who originally proposed the model. The model shown is called a Burgers model. Each element in the middle—i.e., a spring and dashpot arranged in parallel—is called a Kelvin-Voigt unit.

the action of surface tension is also easily determined by a creep test.

Stress relaxation tests

A typical stress relaxation curve will be plotted as a modulus function (G = stress/strain). The curve of $Gf(t)$ (modulus as a function of time) is an exponential decay, and there is no equivalent to the recoil part of the creep test unless another step is programmed. The model fitting routines involve Maxwell elements (Barnes, 2000; Ferry, 1980) that comprise a spring and a dashpot in series, such that they both experience the same stress. Each Maxwell element has a relaxation time associated with it, as well as a modulus (or its inverse, compliance, represented as a spring) and a viscosity (dashpot). Rather than there being a single model equivalent to the Burgers model for fitting stress relaxation data, a series of Maxwell elements is generally used giving a relaxation spectrum.

A disadvantage of the stress relaxation technique is the fact that the signal is always decaying until it is indistinguishable from the noise. If this occurs before the sample reaches steady state, then the test is difficult to analyze. In both step-change techniques, one is interested in generating information over a very wide range of time frames. Thus, the first second of data from either technique is representative of the high-frequency data from a frequency sweep (mechanical spectrum). The steady-state information is, of course, connected to the very-low-frequency data from a sample, and it is often more convenient to access this information via a step-change test than to physically set a very low frequency and wait for the data. As a final point, the mathematical manipulation required to obtain a zero shear viscosity and/or J_e^0 is significant if stress relaxation is the source data (Ferry, 1980).

The choice of strain step to use is guided by the same principles as the choice of stress to use in a creep test. Ideally the strain chosen should come from known data gathered using an oscillation test to define the linear region. Sometimes a particular strain is chosen from known application data, and therefore may be outside the linear response of the sample. When dealing with a torque transducer, there is a fixed dynamic range to consider. The choice of geometry size and transducer range is therefore important in order to avoid overloading the transducer. A stiff sample and too large a geometry will result in a high torque. If a sample is not rigid, the output signal may be too close to the noise.

Transducers that use torsion bars or springs of known compliance may also oscillate at the beginning of the test as the step is imposed. This ringing is symptomatic of the detector and not the sample response, and thus needs to be removed by signal filtering. In a creep test this kind of behavior only occurs if the sample is

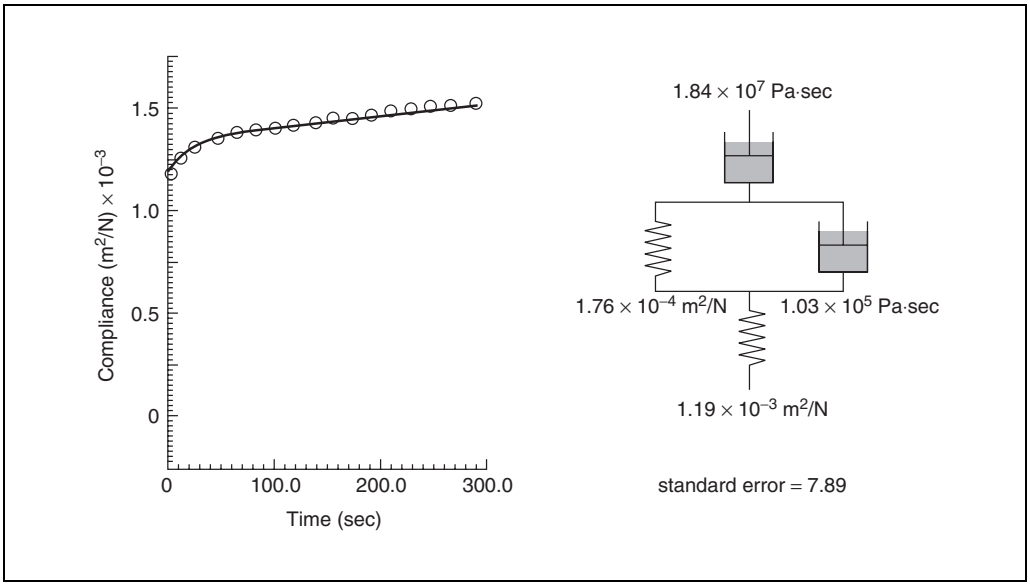

Figure H3.3.5 The creep response of a food (circles) was fitted to a Burger model with one Kelvin-Voight unit. The goodness of fit is shown as the continuous curve and the standard error. The values of compliance and viscosity of the respective springs and dashpots were outcomes of the fitting process.

"ringing." Analysis of this behavior can give insight into the elasticity of the sample.

Critical Parameters and Troubleshooting

A general point to remember when dealing with step change tests when compared to oscillation tests is that the linear response of a sample is quite often different for the two types of test. This is because the physical displacement of the structure of the material is much smaller in an oscillation test, with its forward and backward motion. A step change test is unidirectional and thus may cause more dislocation of a molecule, droplet, or particle, which in turn results in greater "damage."

Noisy data are generally undesirable and should prompt the investigator to try higher values of stress or strain. Remember that the data may be noisy to begin with in a creep test because the sample is barely responding, but as the test progresses the data often leave the noise far behind. Thus, a final curve may be generated that is still easy to analyze. Noise in a stress relaxation test should also prompt the investigator to increase the strain step, although the dynamic range of the transducer may limit the ability to recover steady-state data.

A good diagnostic for creep and stress relaxation tests is to plot them on the same scales as a function of either compliance (J) or modulus (G), respectively. If the curves superimpose, then all the data collected is in the linear region. As the sample is overtaxed, the curves will no longer superimpose and some flow is said to have occurred. These data can still be useful as a part of equilibrium flow. The viscosity data from the steady-state part of the response are calculated and used to build the complete flow curve (see equilibrium flow test in *UNIT H1.2*).

As tests are performed at larger stresses and strains, the results may show decreasing compliance or increasing modulus, respectively. If this is the case, there is a problem with the sample. Generally the cause of this behavior is time dependency. The sample is rebuilding structure and becoming more rigid as subsequent tests are performed. It is very important that the sample be allowed to reach equilibrium before performing step-change tests. Typically, the shape of the curve is distorted at medium to long times because the sample has built up structure during the test. Occasionally a negative stress or strain will appear as internal rearrangements of structure manifest themselves during the test. Again, this is indicative of the test being initiated before the sample is at equilibrium.

In general, it is important to choose a measurement geometry that optimizes sensitivity while minimizing artifacts due to slip. Parallel plate geometry is often the preferred choice as it is relatively easy to cut samples into discs. For liquids or melts, cone and plate or even concentric cylinders may be used, but they are potentially more invasive during sample loading. Care should be taken to allow all stresses from loading to dissipate before starting the test. Normal force sensors are often useful in this regard, or an oscillation time sweep can provide similar guidance.

Anticipated Results

The shape of the creep and recoil curves provide insight about the sample, as does the stress relaxation response. A very strongly curved retardation response resembling a square wave is indicative of dominantly elastic behavior. A stress relaxation response for an elastic solid gives an almost horizontal line with very little decay, because almost all the energy is stored. A creep recoil curve that almost returns to the initial state of zero compliance is indicative of a very elastic system, because stored energy is almost completely recovered.

A linear output is associated with a liquid-like or viscous response. The retardation response of a Newtonian liquid is a straight line through the origin, where the slope of the line is inversely proportional to the viscosity. A stress relaxation response for a Newtonian liquid is a narrow spike, as energy is dissipated very quickly. Typically the recoil part of a creep curve will be almost horizontal if there is little elasticity in the sample.

Real (viscoelastic) materials give an intermediate response that is an exponential curve. The exponential time constants associated with the curve are used to approximate the relaxation times of the material itself. Thus, the shape of the output curve is analyzed to give viscoelastic information, although this model fitting is only strictly legitimate in the linear viscoelastic region. Workers have shown that the mechanical parts of the models (springs and dashpots) can be associated with specific parts of a food's makeup.

Time Considerations

It is clear that these tests take a significant amount of time to perform. Generally, 15 min to 1 hr should be allowed for each test, once the appropriate stresses or strains have been found.

It is advisable to perform replicate tests (three or more) to be sure of reproducibility. For some solid-like materials with long relaxation times, the loading process may take as long as the test.

Literature Cited

Barnes, H.A. 2000. A Handbook of Elementary Rheology. Institute of Non-Newtonian Fluid Mechanics, University of Wales, Aberystwyth, U.K.

Ferry, J.D. 1980. Viscoelastic Properties of Polymers, 3rd ed. John Wiley & Sons, New York.

Key References

Barnes, 2000. See above.

Barnes, H.A., Hutton, J.F, and Walters, K. 1989. An Introduction to Rheology. Elsevier, New York.

Ferry, 1980. See above.

Steffe, J.F. 1996. Rheological Methods in Food Process Engineering, 2nd ed. Freeman Press, East Lansing, Mich.

These texts deal with creep and stress relaxation in some detail and provide a good background to the interested reader. However, some are difficult to obtain (Ferry, 1980, is out of print).

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Determination of Total Phenolics

The phenols or phenolics in wine are important to both red and white wines. In red wines, this class of substances contributes to the astringency, bitterness, and other tactile sensations defined as structure or body, as well as to the wine's red color. In white wines, higher levels of phenolics are generally undesirable, as they contribute to excessive bitterness and to the tendency of the wine to brown when it is exposed to air. Phenolics in grapes and wines include many different substances: phenolic acids (e.g., hydroxybenzoic acids such as gallic acid, hydroxycinnamic acids found in grape juice), three classes of flavonoids found in the skins and seeds (the red anthocyanins, the flavonols, and the abundant flavan-3-ols, which comprise the monomeric catechins), oligomeric proanthocyanidins, and polymeric condensed tannins. (For details on phenolics classes and compound structures, refer to *UNITS I1.2 & I1.3*.) White wine is made by immediately pressing off the skins and seeds after harvesting, and thus contains only small quantities of flavonoids. In contrast, red wine is a whole-fruit extract made by fermenting with the skins and seeds, and the alcohol thus produced is an excellent solvent for these substances.

Measuring these different substances and reporting meaningful values in a single number is an analytical challenge. There are many different procedures for analyzing different classes of phenolic substances, but few are used in wine analysis except for anthocyanin or color measures. HPLC methods (*UNIT I1.3*) that give specific information on individual substances are not widely used in wineries, but are becoming more common as the significance of particular phenolic substances becomes better understood.

There are two widely used methods for the analysis of total phenolics in wine. The Folin-Ciocalteau method (Basic Protocol 1 and the Alternate Protocol) has the advantage of a fairly equivalent response to different phenols, with the disadvantage of responding to sulfur dioxide and sugar. The direct spectral absorbance analysis (Basic Protocol 2) is quick and simple, making it suitable for process monitoring. This method, however, responds differently to the various phenolic classes, making comparisons between different wine types problematic, and also gives significant interference for sorbate.

Wine, of course, is not the only food that contains phenolics. Phenolics are found in all foods, though at low levels in most. Notable foods that are high in phenolics include coffee and tea, chocolate, fruits and derived products, some oils, spices, and some whole grains. Although the following methods were developed for—and first applied to—analysis of wines and grapes, they can be adapted for other foodstuffs (also see Commentary).

DETERMINATION OF TOTAL PHENOLICS BY FOLIN-CIOCALTEAU COLORIMETRY

BASIC PROTOCOL 1

Folin-Ciocalteau (FC) colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. Singleton adapted this method to wine analysis (Singleton and Rossi, 1965) and has written two major reviews on its use (Singleton, 1974; Singleton et al., 1999). The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The FC method has been adopted as the official procedure for total phenolic levels in wine; the Office International de la Vigne et du Vin (OIV), the one international body that certifies specific procedures for wine analysis, accepts the FC method as the standard procedure for total phenolic analysis (OIV, 1990). An earlier variation was the Folin-Denis procedure, but the FC method has displaced it except in a few historical cases of official procedures that have not been updated (AOAC International, 1995).

Contributed by Andrew L. Waterhouse
Current Protocols in Food Analytical Chemistry (2002) I1.1.1-I1.1.8

Color development is slow but can be accelerated by warming the sample. With excessive heating, however, subsequent color loss is quite rapid, and timing the colorimetric measurement becomes difficult to reproduce. The reagent is commercially available, but can be prepared (Singleton and Rossi, 1965). The resulting solutions are treated as hazardous waste, and the scale of the original procedure creates a lot of waste. Fortunately, modern liquid-measuring equipment now allows for microscaling the reaction to the volume of a UV-Vis cuvette, reducing the cost of the reagent and waste disposal (see Alternate Protocol).

Materials

Sample, e.g., white wine or 10% (v/v) red wine in water
Gallic acid calibration standards (see recipe)
Folin-Ciocalteau (FC) reagent (Sigma; also Singleton and Rossi, 1965), stored in the dark and discarded if reagent becomes visibly green
Sodium carbonate solution (see recipe)

100-ml volumetric flask
Spectrophotometer set to 765 nm, with 1-cm, 2-ml plastic or glass cuvettes

1. Place 1 ml sample, a gallic acid calibration standard, or blank (deionized or distilled water) in a 100-ml volumetric flask.

   Samples and standards should be analyzed in triplicate.

   If any sample has an absorbance reading above that of the 500 mg/liter standard, it must be diluted adequately and remeasured. White wine can typically be analyzed without dilution. Red wine must be diluted with water (usually ten-fold) to fall into the range of the standards.

2. Add ~70 ml water, followed by 5 ml FC reagent. Swirl to mix and incubate 1 to 8 min at room temperature.

   The incubation must not be >8 min (see Critical Parameters, discussion of reaction time and temperature).

3. Add 15 ml sodium carbonate solution.

4. Add water to the 100-ml line, mix, and incubate 2 hr at room temperature.

5. Transfer 2 ml to a 1-cm, 2-ml plastic or glass cuvette and measure its absorbance at 765 nm in a spectrophotometer.

6. Subtract the absorbance of the blank from all readings and create a calibration curve from the standards.

7. Use this curve to determine the corresponding gallic acid concentration of the samples. Be sure to multiply by any dilution factor for the correct concentration (i.e., by ten for red wines). Report values in gallic acid equivalents (GAE) using units of mg/liter (see Critical Parameters, discussion of standardization).

ALTERNATE PROTOCOL

MICROSCALE PROTOCOL FOR FOLIN-CIOCALTEAU COLORIMETRY

This protocol is adapted for small sample volumes. The reaction is performed directly in a 2-ml cuvette. For a list of materials needed, see Basic Protocol 1.

1. Put 20 µl sample, a gallic acid calibration standard, or blank (deionized or distilled water) into a 1-cm, 2-ml plastic or glass cuvette.

2. Add 1.58 ml water, followed by 100 µl FC reagent. Mix thoroughly by pipetting or inverting and incubate 1 to 8 min.

   The incubation must not be >8 min (see Critical Parameters, discussion of reaction time and temperature).

3. Add 300 µl sodium carbonate solution, mix, and incubate 2 hr at room temperature.

   A final volume of 2 ml must fill the cell adequately for a reading.

4. Measure sample absorbance at 765 nm and analyze as described (see Basic Protocol 1, steps 6 to 7).

DETERMINATION OF TOTAL PHENOLICS BY SPECTRAL ANALYSIS

BASIC PROTOCOL 2

Phenolic substances all absorb UV light, and all of them have some absorbance at 280 nm. This property can be used to determine phenolics by spectral analysis. One problem with this method is that each class of phenolic substances has a different absorptivity (extinction coefficient, e) at 280 nm. Thus, the results cannot be related to any specific standard and are reported directly in absorbance units (AU). This also means that disparate wines (or other disparate samples) are difficult to compare with this method, as they are likely to have very different compositions.

The value of this method is that it is extremely simple and rapid, requiring only filtration and, in some cases, dilution. It is very suitable for monitoring wines during various stages of processing (e.g., fermentation) and for comparing similar wines (e.g., a single grape variety from different vineyards, or wines from a particular vineyard over different vintages).

Materials

Sample, e.g., red or white wine
Filter membrane, e.g., polytetrafluoroethylene (PTFE)
Cuvettes, transparent at 280 nm (e.g., quartz or methacrylate)
Spectrophotometer, set to 280 nm

1. Filter a sample or blank (deionized or distilled water) with a PTFE filter membrane or other material to achieve clarity.

   Nylon or other membranes that absorb phenolics should not be used. Membranes can be tested for phenolic absorption by comparing absorbance after single and double filtration.

2. Transfer an appropriate volume of sample to a quartz or methacrylate cuvette and measure absorbance at 280 nm in a spectrophotometer. If absorbance is not within the acceptable precision of the spectrophotometer (usually $A < 2$ AU), dilute sample as necessary and repeat.

3. Subtract absorbance of blank, and correct absorbance to original concentration and a 1-cm cuvette path length. Subtract 4 AU to report final value.

   For instance, if a sample is diluted ten-fold with water and a reading of 0.85 AU is observed with a 2-mm cell, the correction would be as follows:

   total phenol = [A280 × DF × (1 cm/b)] - 4

   = [0.85 × 10 × (1 cm/0.2 cm)] - 4 = 38.5 AU

   where DF is the dilution factor, b is the cell path length, and 4 is an arbitrary correction for nonphenolic absorbance (see Critical Parameters, discussion of spectral analysis).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gallic acid calibration standards

Dissolve 0.5 g gallic acid in 10 ml ethanol and then dilute to 100 ml with water (5 g/liter final). Dilute 1, 2, 5, and 10 ml to 100 ml with water to create standards with 50, 100, 250, and 500 mg/liter concentrations, respectively. Store up to 2 weeks at 4°C.

Standards will retain 98% of their potency for 2 weeks if kept closed under refrigeration (4°C), but this potency is retained for only 5 days at room temperature.

Commercial gallic acid is usually adequately pure, but can be recrystallized from water if desired.

Sodium carbonate solution

Dissolve 200 g anhydrous sodium carbonate in 800 ml water and bring to a boil. After cooling, add a few crystals of sodium carbonate and let sit 24 hr at room temperature. Filter through Whatman no. 1 filter paper and add water to 1 liter. Store indefinitely at room temperature.

COMMENTARY

Background Information

There are many phenolic substances in plants and thus in foods. Rich dietary sources of phenolics include fruits, tea, coffee, cocoa, and processed foods derived from these, such as wine. At high levels, and in particular when sugar levels are low, phenols impart an astringency, bitterness, and color to foods. In red wine, unsweetened tea, and chocolate products, the taste is heavily influenced by the presence of phenolics. Therefore, an assessment of phenolic content in food is of great importance.

Folin-Ciocalteau method

The Folin-Ciocalteau (FC) procedure is one of the standard procedures in wine analysis, as well as in tea analysis (Wiseman et al., 2001). One drawback in interpretation is that different classes of phenolics have varying taste attributes, and tests for chemical astringency based on precipitation of proteins have been recently developed (Adams et al., 1999). In addition, if the food product contains sugar, it can mask the bitterness and astringency, as observed in ripe fresh fruit, sweetened chocolates, and tea.

The differential sensory effect of phenolics aside, a major advantage of the FC procedure is that it has a fairly equivalent response to different phenolic substances in wine, making it suitable for measuring accurate mass levels of total phenolic substances. Among the abundant phenolics in wine, the mass response factor relative to gallic acid ranges from 0.87 for caffeic acid to 1.10 for epicatechin based on values from Singleton (1974). The glucosides give lower values, but their mass is increased by the nonphenolic glycosidic substituent. Their response appears to be similar on a phenolic fraction basis. Monohydroxyphenolics such as coumaric acid also give low values, but these constitute a small fraction of the phenolics in wine. In general, the response of a phenolic is due to the number of phenolic groups, and Singleton (1974) describes the controlling factors in detail.

The FC method has also been applied to other foods. One example of particular use is for analysis of tea (Wiseman et al., 2001). The method has also been applied to vegetables (Kaur et al., 2002) and fruit (Pearson et al., 1999; Vinson et al., 2001), although in one instance the only corrected interference was ascorbate. For analysis of foodstuffs other than wines and grapes, the analyst must be aware of potential interferences. In other fields, the method has been used for analysis of medicines (Sadler and Jacobs, 1995), trees in wood chemistry (Yu and Dahlgren, 2000), and fresh waters (Thoss et al., 2002).

The FC procedure was automated some time ago (Slinkard and Singleton, 1977). Although there have been no more published reports on contemporary automation, there are many laboratories that have adapted the procedure to clinical analyzers (G. Burns and T. Collins, pers. comm.). It seems likely that the procedure could be adapted to other analyzers as well.

There are few direct comparisons of total phenol values and antioxidant measurements, although some do exist (e.g., Baderschneider

et al., 1999). In general, the response of total phenol tests is comparable to antioxidant tests, with better correlations for antioxidant tests based on aqueous systems as opposed to those based on lipid media.

Spectral analysis

Phenolic substances can also be quantified by measuring absorbance at 280 nm. The applicability of this method is far more limited, however, because absorbance properties of different phenolics vary and cannot be related to a specific standard. Because of this, and because of the extreme ease of the method, spectral analysis is well suited for using total phenolic content for process monitoring.

Sample preparation

The preparation of extracts from solid foods is not trivial. In general, 70% (v/v) acetone is used to extract proanthocyanins and condensed tannins, and aqueous methanol is typically used for other classes of phenolics (*UNIT F1.1*). However, the acid levels used in some reported extraction procedures can have a devastating effect on the recovery of particular flavonoids, and this has been carefully studied for HPLC analysis of flavonoids (Merken et al., 2001). Although some transformations simply hydrolyze glycosides, which would not significantly change the total phenolic content, other degradations may significantly decrease the amount of phenolics present. In the absence of a well-developed procedure for the extraction of a particular compound from a similar matrix, re-extractions and recovery must be validated for any extraction protocol. For solid foodstuffs, the final results would be expressed in mg/100 g or mg/kg.

Critical Parameters

Folin-Ciocalteau method

Interferences. Because the color formation of the Folin-Ciocalteau reaction is based on chemical reduction of the reagent, this reaction is general enough to allow for interference from a number of sources. In wine, the principal interfering compounds are sulfur dioxide and ascorbate, though high levels of sugar indirectly enhance the readings of other analytes. Because there can be many other interferences in non-wine samples, however, it is necessary to thoroughly investigate the use of this method for different samples. The most problematic interference may well be sugar, as it is not mentioned in the original report (Singleton and Rossi, 1965), but can be found at very high levels in, for instance, fruits. Another non-wine issue comes from the fact that the Lowry method for protein analysis is based on the reaction of the FC reagent with tyrosine phenolic groups, so samples with high protein levels will not be suitable for phenolic analysis by FC. In addition, it should be noted that phenolics will interfere with protein analysis by the Lowry method. Some investigators have applied the use of the FC procedure to analyze phenolic levels in human blood fractions, such as plasma (Nigdikar et al., 1998). It is likely, however, that this method will respond to changes in the large amount of constitutive redox-active substances, such as ascorbate, urate, and tocopherol, as well as any proteins, making it difficult to attribute changes in response to the appearance of phenolics in the blood.

The FC method does not respond to sulfur dioxide alone, but does respond to sulfur dioxide in the presence of phenolic compounds. Presumably, the phenols are oxidized by the FC reagent and then reduced by the sulfur dioxide, creating an interfering response by a type of catalytic cycle. Unfortunately, the magnitude of the interference is not constant (Saucier et al., 1999), so it is not possible to suggest an accurate correction factor, though approximate mass correction factors of 0.1 to 0.2 have been suggested (thus, 10 mg/liter sulfur dioxide would yield a response of 1 to 2 mg/liter in the FC assay; Singleton, 1988). Generally, the case where the sulfur dioxide interference is significant is in white wines, which have a lower range of total phenolic levels and a high level of sulfur dioxide. It has been suggested that this interference renders the method unusable (Somers and Ziemelis, 1980), but that conclusion has not been accepted by others.

Ascorbate is present at very low levels in wine unless it has been added, which is legal but rarely done in the United States, although it is common in some other countries. In other food samples, especially fresh fruits, ascorbate levels can be very high. For kiwifruit, a correction of 1 mg/liter per 1 mg/liter ascorbate must be applied.

The interference of sugar is easily corrected, and is only necessary with sweet or semisweet wine (>2% [w/v] sugar). With fruit samples, the sugar levels can be very high, and it is not clear if adequate corrections can be applied. Generally, it is not advisable to analyze must for phenolic levels by the FC method because of the complexity of the sugar correction. Sin-

Table I1.1.1 Approximate Correction to Folin-Ciocalteau Results for Wines Containing Invert Sugar[a]

Apparent phenol content (mg GAE/liter)	Invert sugar content		
	2.5%	5.0%	10%
100	5	10	20
200	10	15	40
500	20	30	50
1000	30	60	100
2000	60	120	200

[a]The correction value should be subtracted from the apparent phenol content for an accurate value. This correction applies to analyses conducted at room temperature. Abbreviation: GAE, gallic acid equivalents. Reproduced from Slinkard and Singleton (1977), with permission from the American Society for Enology and Viticulture.

gleton suggests conducting the analysis of the standards with the same level of sugar as the sample, but this is a complex issue because different sugars yield different intereferences (i.e., fructose has a higher response than glucose). Singleton suggests the corrections in Table I1.1.1 when sugar is not added to standards (Slinkard and Singleton, 1977) and describes additional correction factors for warmer temperatures.

These correction factors have not been directly applied to other foods, but it would be prudent to test for the presence of interfering substances, in particular sugars and ascorbate in fresh fruits, and to assess their level of interference in the assay. Correction factors for other interferences may need to be specifically developed.

Limits of detection and quantitation. Because wines that have total phenol levels lower than 50 mg/liter are quite rare, this is not a significant issue for wine. For other sample types, however, a limit of quantitation of ~0.027 AU or 20 mg/liter would be expected, based on a sample-to-sample variance of 0.003 AU (Singleton and Rossi, 1965).

Standardization. Because the analysis reveals the presence of many different substances in one result (in the case of wine, this could mean thousands of substances if one takes into account the diversity of proanthocyanidins), the only practical standard is a single substance. In the cases of wine and tea, the accepted standard is gallic acid. It is a particularly good standard because it is relatively inexpensive in pure form and is stable in its dry form. Other substances have been used, and in principle any phenol could be used, but gallic acid is strongly recommended for the above reasons and the fact that the use of a single standard makes it easier to compare data.

Gallic acid is quite stable in dry form, but will oxidize once it is in solution. This reaction is enhanced at higher temperatures. The author has found that the standard solutions should be stored in full or nearly full bottles that are kept tightly sealed between uses and are stored under refrigeration. The author has observed that >5% potency is lost after ~1 week at room temperature, but this same loss takes ~2 weeks with refrigeration. The authors would expect that greater air exposure will also accelerate decomposition.

Because a single substance is used, the result must be reported as a response equivalent to the amount or concentration of that substance. As wine is analyzed on a concentration basis, the result is reported in gallic acid equivalents (GAE) using units of mg/liter. For any standard, the results must always be reported on an equivalent basis to avoid the perception that one is measuring the amount of the standard substance.

Reaction time and temperature. The oxidative reaction caused by the FC reagent is slow and is not complete when the reading is taken. In addition, the colored product is unstable. The measurement is based on the kinetics of the process. Thus, the time of the colorimetric reading is most critical, and the temperature will affect the extent of the reaction and degradation. This is one of the reasons that standards are run each time, to accommodate for changes in room temperature, timing, and reagent condition. At higher temperatures, the colored product has a very limited lifetime. For a full discussion, see Singleton et al. (1999).

The FC reading time can be accelerated by heating the solution and halving, as a rule of thumb, the reaction time for each temperature increase of 10°C. Thus, at 40°C, readings can be taken after 30 min. Decreasing the time

further by higher temperatures becomes problematic for manual measurements because the timing of each reading becomes more critical with each increase in temperature. The degradation of the component that absorbs at 765 nm, in particular, becomes very rapid at higher temperatures, so analyses that run just slightly too long will yield very poor results (Singleton, 1999). Thus, 40°C is usually the highest recommended temperature for manual use, but higher temperatures are described for automated analyses (Slinkard and Singleton, 1977).

Precision. Because the FC method relies on reaction kinetics and not stoichiometric conversion, it is not very precise, and variations of ~5% are typical for replicates, depending on the temperature control and timing precision of the reagent additions and spectral measurements.

Spectral analysis

Interferences. In wine the principal interference is sorbic acid. This is a preservative typically added to sweetened wines that have residual sugar. Because yeast would continue to ferment this sugar, spoiling the product in the bottle, such wines must be rendered sterile at bottling. This is usually done by sterile filtration, sometimes combined with the addition of sorbic acid. Sorbic acid has a very strong absorbance at 280 nm and will result in exceptionally high readings (up to 36 AU). There appears to be some dispute about the ease of dealing with the interference, as the author of this method claims that it is easy to remove the sorbic acid by isooctane extraction (Somers and Ziemelis, 1985). It has been reported, however, that an impractically large volume of isooctane would be needed to remove the sorbate (Tryon et al., 1988). Although it is likely that serial extractions would be very effective, routine multiple extractions of all samples would make the method significantly more complex when analyzing unknown wines that may contain sorbate.

Background. The method also attributes 4 AU to nonphenolic substances in all samples, and so this value is subtracted to reflect the true absorbance due to phenols. The origin of this background absorbance is not clear, but is thought to be due to protein and nucleotides. The magnitude of the background absorbance does vary with a standard deviation of 1 AU (Somers and Ziemelis, 1985). Thus, for typical white wines with an uncorrected absorbance of 8 AU, the subtraction of 4 AU leaves 4 AU with an expected standard deviation of 1 AU or 25% due to the variance in the correction factor.

Standards. There is no standard or calibration with standards, so these issues are moot.

Anticipated Results

The level of total phenolics in white wines varies from ~100 to 300 mg/liter by the FC method. The levels will be on the low end of the scale if the must was subjected to oxidative treatment and the pressing was very light. Higher levels will be observed when harder pressing of the solids is utilized or if the wine was aged in new oak barrels. By spectral analysis, white wines have an average corrected absorbance of 4 AU, with a range of 1 to 11 AU.

The FC results will be compromised by high levels of sulfites, but because sulfite levels are almost always measured in wines, the possibility of sulfite interference in a wine should be anticipated. The spectral method will be compromised by the presence of sorbate. In known wines this can easily be anticipated and, because its use is limited to sweet wines, it may be possible to check for it selectively. Another clue to its presence would be a very high absorbance. Sorbate can also be measured in wine (Caputi and Stafford, 1977).

Red wines have total phenolic levels of 1 to 3 g/liter, with typical average of ~1.8 g/liter. Differences are due to differing amounts of phenolics in grapes based on variety and growing conditions, with moderate to cooler climates yielding higher levels. Production techniques can have a secondary effect, and longer contact times or higher temperatures will increase the amount of phenolics extracted from the grape solids into the wine. Red wines have a reported range of 23 to 100 AU, with an average of 54 AU. Because levels are so high, interferences other than sorbate do not introduce significant error in the spectral method.

Time Considerations

When the FC analysis is carried out manually, there are limits to the number of samples that can be handled at once because of the need to time the reagent mixing and spectral readings. In the author's laboratory, single analysts can run 20 samples per day, divided into 2 sets. Since each sample is run in duplicate and 4 standards are included in each run, 50 individual results are generated in a day.

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The original description for the use of the FC method for wine analysis.

Singleton et al., 1999. See above.

A thorough review of the FC method's use in wine analysis by the original author.

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The key compilation of spectral methods.

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Extraction and Isolation of Polyphenolics

Polyphenolics constitute a wide range of chemical compounds composed of aromatic ring(s) with one or more hydroxyl substituents, including their functional derivatives. Methods for extraction and isolation of polyphenolics from plant material are described in this unit. Extraction and isolation are the first important steps for separation, characterization, and quantification of polyphenolics from plant material. Polyphenolics are often most soluble in organic solvents less polar than water. The solubility is dependent on the polar properties of the polyphenolics. The correct selection of the extracting solvent is not as simple as it may seem. Aqueous methanol is a popular choice of solvent (see Background Information).

Basic Protocol 1 describes the extraction of polyphenolics from freeze-dried powdered plant material using aqueous methanol and an ultrasound bath. The cavitation produced by ultrasound when used in aqueous methanol extraction assists to increase the mass transfer rate and allows higher product yield with reduced extraction time and solvent usage. Alternate Protocol 1 describes a simple sample preparation for the extraction of polyphenolics from fresh raw plant material using methanol and homogenization. Basic Protocol 2 describes an effective solid-phase extraction for isolation/purification of polyphenolics into non-anthocyanin and anthocyanin fractions. Interfering compounds such as sugars and organic acids are eliminated in this procedure. Alternate Protocol 2 describes the fractionation of polyphenolics into neutral and acidic fractions based on the fact that polyphenolic acids are completely ionized at pH 7.0 and un-ionized at pH 2.0.

ULTRASOUND-ASSISTED AQUEOUS METHANOL EXTRACTION OF POLYPHENOLICS

BASIC PROTOCOL 1

In this method, 80% aqueous methanol and ultrasound are used to extract polyphenolics from freeze-dried powdered plant material. Ultrasound-assisted extraction is a rapid and efficient method for the extraction of polyphenolics. Low-frequency, high-energy, high-power ultrasound in the kHz range has the advantage of significantly reducing extraction time and enhancing extraction yield. The use of fine powdered plant material maximizes polyphenolics extraction by increasing the surface area of the sample and facilitating the disruption of biological cell walls.

Materials

Freeze-dried powdered plant material, ground with a blender (e.g., Waring) or laboratory mill (e.g., Thomas-Wiley) and stored at −18°C under nitrogen gas, protected from light
80% (v/v) aqueous methanol
Nitrogen gas
Absolute methanol

500-ml Erlenmeyer flask
Ultrasonic cleaning bath
Whatman no. 2 filter paper
Buchner funnel
1000-ml round-bottom evaporating flask
Rotary evaporator with water aspirator or vacuum pump, 40°C water bath

CAUTION: Sonication, filtration, and transfer should be performed in a fume hood. Personal protection equipment—laboratory coat, gloves, and safety glasses—must be used. Ear protection should be utilized during sonication.

Contributed by Dae-Ok Kim and Chang Y. Lee
Current Protocols in Food Analytical Chemistry (2002) I1.2.1-I1.2.12

1. Mix 10 g of ground freeze-dried samples (accurately weighed) with 100 ml of 80% aqueous methanol in a 500-ml Erlenmeyer flask.

   The powdered plant material maximizes polyphenolic extraction because of its high contact area with solvent and easy destruction of biological cell walls. Ethanol may be employed instead of methanol in routine extractions to avoid the toxic properties of methanol. The main advantage of methanol over ethanol is its lower boiling point.

2. Under subdued light, immerse the flask into the ultrasonic bath and sonicate for 20 min at room temperature with continual nitrogen gas purging and periodic shaking. Do not allow the temperature to rise.

   To prevent oxidation of polyphenolics during extraction, a constant stream of nitrogen gas is introduced into the Erlenmeyer flask to provide an oxygen-free environment. The temperature in the ultrasonic bath during solvent extraction should be maintained at room temperature or lower because cavitational effects are reduced and possible degradation of polyphenolics takes place at increased temperatures. The temperature in the ultrasonic bath can be controlled by cooling systems or ice. The intense ultrasound achieves higher production yields, despite reduced contact time and solvent consumption, due to enhanced mass transfer rate.

3. Filter the mixture through Whatman no. 2 filter paper by vacuum suction using a chilled Buchner funnel. Rinse the filter cake with 50 ml absolute methanol.

4. Re-extract the residue with 100 ml of 80% aqueous methanol by repeating steps 2 and 3.

   Two extractions are often satisfactory for most plant material.

5. Transfer filtrates to a 1000-ml round-bottom evaporating flask with 50 ml of 80% aqueous methanol.

6. Evaporate methanol in a rotary evaporator under vacuum at 40°C until the volume of extract is reduced to 10 to 30 ml.

   Solvent evaporation should be done under reduced pressures at low temperatures to minimize the degradation of extracted polyphenolics. Due to the likely occurrence of hydrolysis, isomerization, and polymerization at higher temperatures, temperatures for the evaporation process should be maintained at 40°C or below.

7. Resolubilize the concentrate to a 100-ml volume with deionized distilled water. Flush with nitrogen gas to prevent oxidation. Store at –4°C until analysis (up to 1 day).

ALTERNATE PROTOCOL 1

HOMOGENIZER-ASSISTED METHANOL EXTRACTION OF POLYPHENOLICS

In this alternative procedure, absolute methanol rather than aqueous methanol is used to extract the polyphenolics from fresh plant material instead of freeze-dried samples. Raw plant material is washed, dried, and then immediately extracted. This protocol employs a blender to first macerate the plant material to increase the sample surface area for better contact of the solvent and the sample.

Additional Materials *(also see Basic Protocol 1)*

Fresh plant material
Ascorbic acid

500-ml beaker
Blender (e.g., Waring Blendor), chilled to 4°C
Polytron homogenizer (or equivalent)
500-ml filter flask

1. Mix 50 g fresh plant material (net weight) with 1 g ascorbic acid and 100 ml absolute methanol in a 500-ml beaker.

   Ascorbic acid is incorporated into the extraction system to prevent oxidation of polyphenolics during extraction. Since fresh plant material contains considerable quantities of water, the use of absolute methanol rather than aqueous methanol provides a more effective extraction.

2. Transfer contents of beaker into chilled (4°C) blender and immediately macerate by blending at high speed for 3 min.

   The speed of homogenization should start slowly and then be gradually increased. This work should be performed in the fume hood using proper personal protection equipment (PPE).

3. Transfer ground/crushed material back into its original beaker with 50 ml of 80% aqueous methanol and place in an ice bath.

4. Homogenize sample in ice bath using a Polytron homogenizer set at 7 for 2 min.

   This work also should be performed in the fume hood using proper PPE. The sample should be kept in an ice bath to prevent an increase of temperature that may cause possible degradation of polyphenolics during homogenization.

5. Filter the homogenous sample through a Whatman no. 2 filter paper into a chilled 500-ml filter flask by water-aspirated vacuum suction using a chilled Buchner funnel. Pour the homogenate slowly into the center of the filter paper and allow to advance to the edges before applying the vacuum.

   In the case of extremely viscous samples or samples with high pectin contents, centrifugation is often employed. The sample is centrifuged for 20 min at 12,000 × g, 4°C, and then the supernatant is filtered.

6. Transfer the residue from the filter paper back to the Polytron homogenizer with 100 ml methanol and repeat steps 4 and 5. Combine filtrates and discard the extracted plant material.

7. Transfer filtrates to a 1000-ml round-bottom evaporating flask with 50 ml of 80% aqueous methanol and evaporate off methanol in a rotary evaporator under vacuum at 40°C to a volume of 10 to 30 ml.

8. Dissolve the extract to a volume of 100 ml with deionized distilled water. Flush with nitrogen gas to prevent oxidation. Store at −4°C until analysis (up to 1 day).

SEPARATION OF ANTHOCYANIN AND NON-ANTHOCYANIN FRACTIONS

BASIC PROTOCOL 2

Due to the large number of structurally similar polyphenolics in plants, analysis of individual polyphenolics is relatively difficult and complicated. Considerable amounts of interfering material can be extracted with the polyphenolic extraction procedure. An isolation/purification step is often required to eliminate components that may interfere with the analysis. In this protocol, a simple fractionation of the polyphenolic extract is performed using preconditioned C18 Sep-Pak cartridges to separate anthocyanins from non-anthocyanin polyphenolics (Figure I1.2.1; also see *UNIT F1.1*). This fractionation technique, using solid-phase extraction, makes the analysis of individual polyphenolics by high-performance liquid chromatography possible.

Materials

Ethyl acetate
Methanol with and without 0.1% (v/v) HCl
0.01 N aqueous HCl
Aqueous extract (sample) of polyphenolics (see Basic Protocol 1 or Alternate Protocol 1)
Nitrogen gas

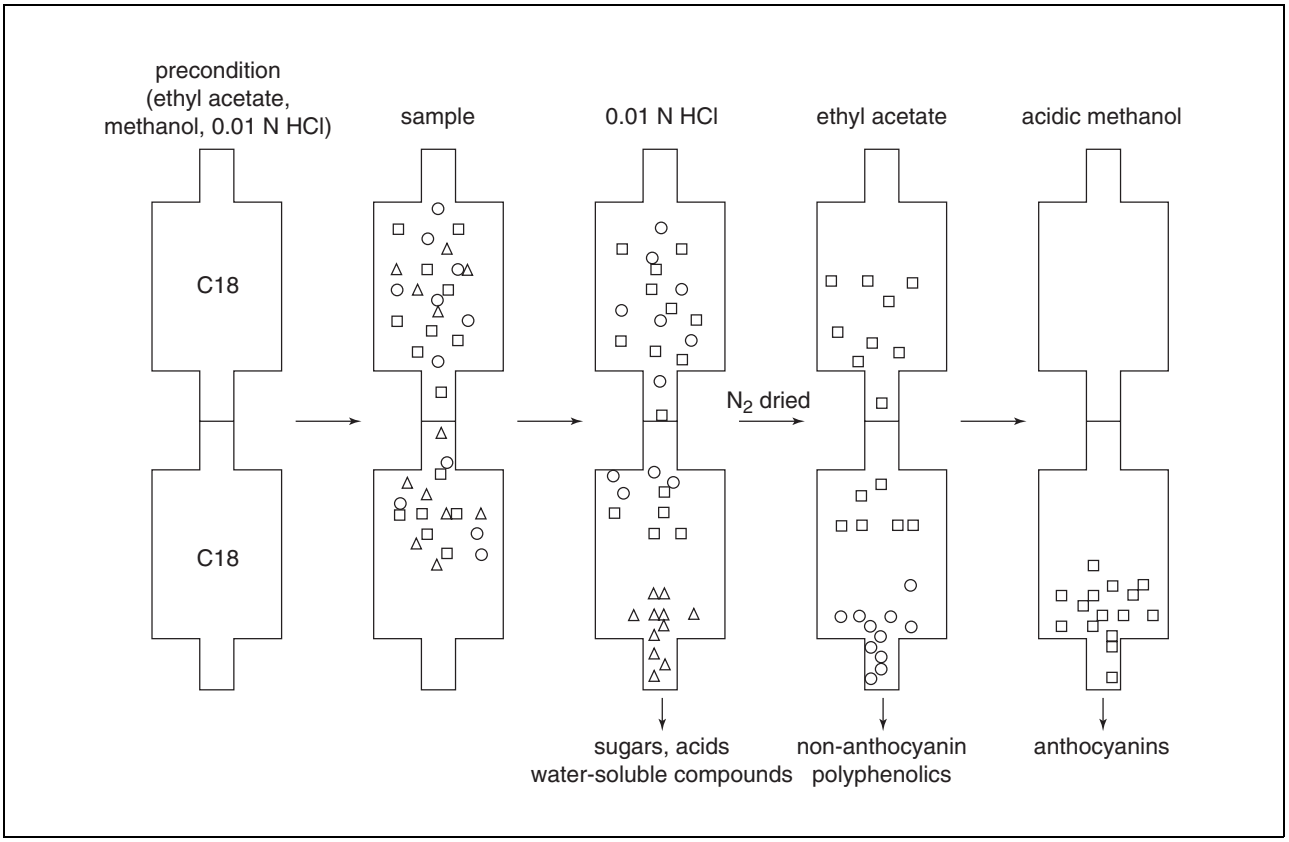

Figure I1.2.1 Fractionation of polyphenolics into non-anthocyanin and anthocyanin fractions using C18 cartridges (circles: non-anthocyanin polyphenolics; squares: anthocyanins; triangles: sugars, acids, and water-soluble compounds).

C18 Sep-Pak cartridges (Waters Chromatography)
0.45-µm polyvinylidene fluoride (PVDF) syringe-tip filter
50- and 100-ml round-bottom flasks
Rotary evaporator with water aspirator or vacuum pump, at 40°C

1. Connect two C18 Sep-Pak cartridges and precondition by sequentially passing 10 ml ethyl acetate, 10 ml absolute methanol, and 10 ml of 0.01 N aqueous HCl through the cartridges.

   A single cartridge can be used depending on solute concentration.

2. Filter aqueous polyphenolic extract (sample) through a 0.45-µm PVDF filter. Load a known volume of filtered extract onto cartridges.
3. Wash cartridges with 6 ml of 0.01 N aqueous HCl to remove sugars, acids, and other water-soluble compounds.
4. Dry cartridges by allowing a current of nitrogen gas to pass through the connected Sep-Pak cartridges for 10 min.
5. Rinse cartridges with 40 ml ethyl acetate to elute polyphenolic compounds other than anthocyanins and collect in a 100-ml round-bottom flask.
6. Elute the adsorbed anthocyanins from the cartridges with 6 ml acidic methanol and collect in a separate 50-ml round-bottom flask.
7. Remove the solvents of the non-anthocyanin fraction and anthocyanin fraction using a rotary evaporator under reduced pressure. Evaporate ethyl acetate at 20°C and methanol at 40°C or under nitrogen.

8. Dissolve each fraction in 5 ml deionized distilled water. Flush with nitrogen gas to prevent oxidation. Store at −4°C until analysis (up to 1 day).

   To obtain a sufficient volume for subsequent analyses, several individual separations may be performed and then combined.

NEUTRAL AND ACIDIC FRACTIONATION OF POLYPHENOLICS

ALTERNATE PROTOCOL 2

In this protocol, polyphenolics are fractionated into neutral and acidic fractions to prevent interference among polyphenolics in HPLC analysis. Phenolic acids are completely ionized at pH 7.0 and un-ionized at pH 2.0. This property allows for fractionation of neutral polyphenolics at pH 7.0 and acidic polyphenolics at pH 2.0. Two individually preconditioned C18 cartridges, one for neutral polyphenolics and the other for acidic polyphenolics, are used for this separation (Figure I1.2.2).

Additional Materials *(also see Basic Protocol 2)*

Methanol
5 N NaOH
0.01 N and 1 N HCl (aqueous)

1. Precondition a C18 Sep-Pak cartridge for neutral polyphenolics with 2 ml methanol followed by 2 ml deionized distilled water.
2. Filter the aqueous polyphenolic extract with a 0.45-µm PVDF filter and adjust the pH to 7.0 with 5 N NaOH.

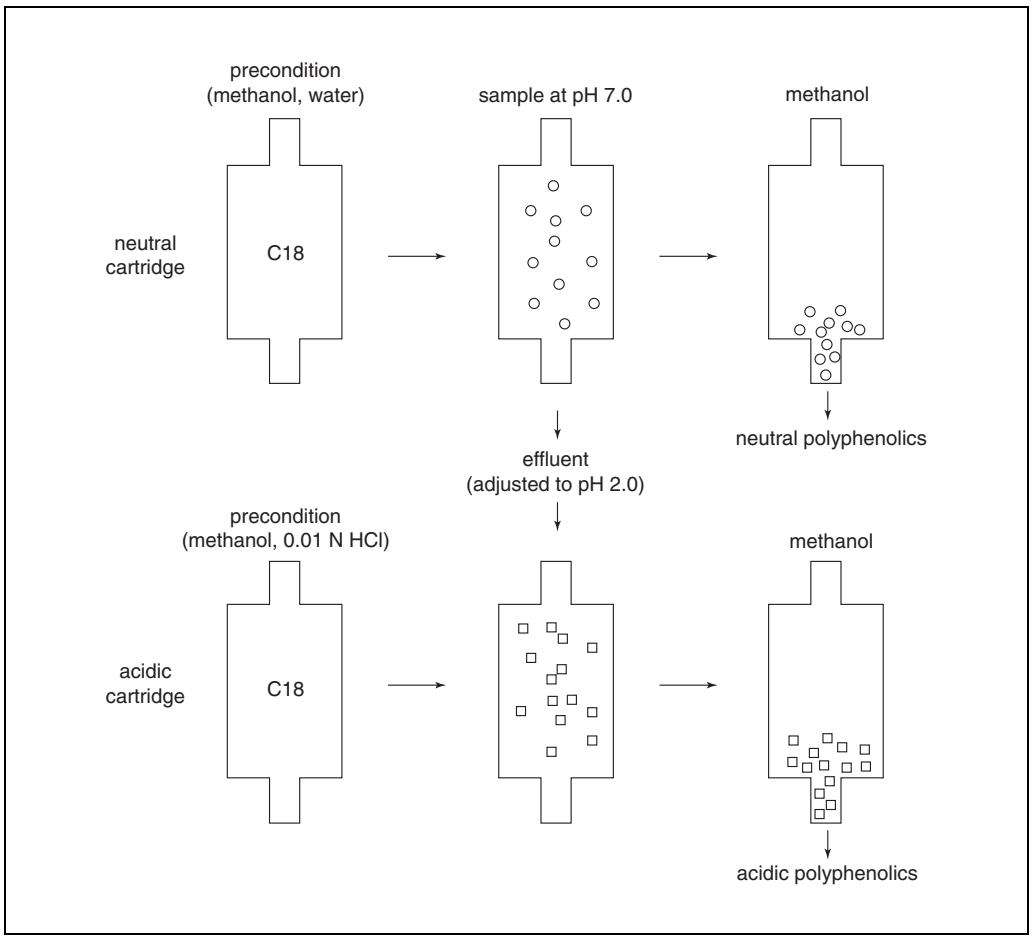

Figure I1.2.2 Fractionation of polyphenolics into acidic polyphenolics and neutral polyphenolics using C18 cartridges (circles: neutral polyphenolics; squares: acidic polyphenolics).

3. Pass extract onto the preconditioned C18 Sep-Pak cartridge to absorb the neutral polyphenolics and collect the effluent in a 50-ml round-bottom flask.
4. Precondition a second C18 Sep-Pak cartridge for acidic polyphenolics with 2 ml methanol followed by 2 ml of 0.01 N HCl.
5. Adjust the pH of the effluent portion from the first cartridge to pH 2.0 using 1 N HCl and pass through the second (acidic) cartridge to absorb the acidic polyphenolics.
6. Elute both neutral and acidic polyphenolics from their respective C18 Sep-Pak cartridges with 5 ml absolute methanol.
7. Remove the solvent of both neutral and acidic fractions using a rotary evaporator under vacuum at 40°C, or under nitrogen.
8. Resolubilize the fractions in 5 ml deionized distilled water. Flush with nitrogen gas to prevent oxidation. Store at −4°C until analysis (up to 1 day).

COMMENTARY

Background Information

Types of polyphenolics

Widely distributed in nature, polyphenolics are important aromatic secondary metabolites of plants. They contribute to the sensory quality (i.e., color, flavor, and taste) as well as the antioxidant activity of fruits, vegetables, beverages, and grains. Generally, fruits have higher polyphenolics than vegetables. Strawberries, raspberries, plums, oranges, and others are known fruits having higher concentrations of polyphenolics. Proteggente et al. (2002) reported that red plums, strawberries, and raspberries are categorized as anthocyanin-rich fruits; oranges and grapefruits are classified as flavanone-rich fruits; green grapes, onions, leeks, lettuce, broccoli, spinach, and green cabbage are flavonol-rich fruits and vegetables; and apples, pears, tomatoes, and peaches are hydroxycinnamate-rich fruits. More than 8000 natural polyphenolics are currently known to occur in plant sources. Flavonoids and their derivatives are the most common and the largest group of polyphenolics, with >4000 known structures (Middleton and Kandaswami, 1994). The term polyphenolics can be defined as compounds composed of aromatic benzene ring(s) substituted with hydroxyl groups, including functional derivatives. Polyphenolics range from structures that are very lipophilic (e.g., tangeretin) to those that are very hydrophilic (e.g., quercetin-3-sulfate). Most phenolic acids and flavonoids are compounds of relatively low molecular weight.

Polyphenolics are classified into three important groups: phenolic acids, flavonoids, and tannins. Phenolic acids include hydroxybenzoic (C_6-C_1), hydroxyphenylacetic (C_6-C_2), and hydroxycinnamic (C_6-C_3) acids (Figure I1.2.3; also see Figure I1.3.3). Hydroxycinnamic acids are most widely distributed in plant tissues. The important hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic

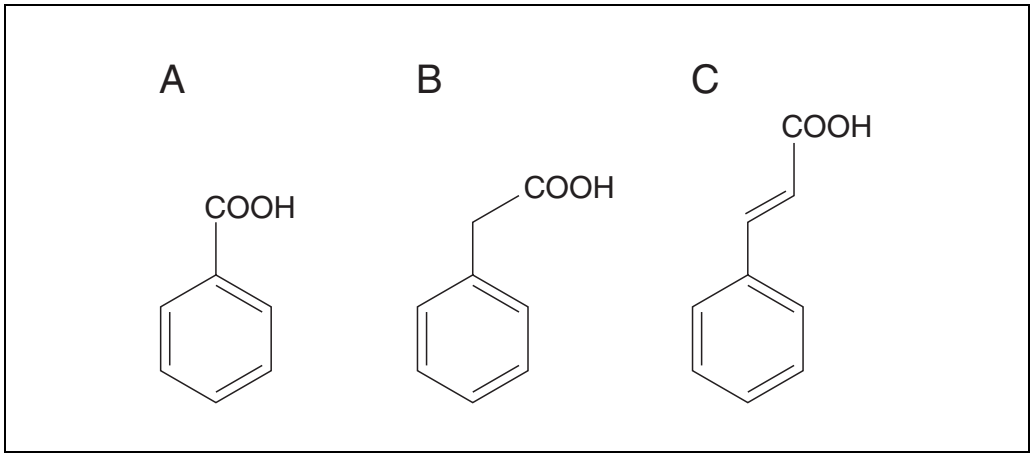

Figure I1.2.3 Structures of the hydroxybenzoic acids (**A**), hydroxyphenylacetic acid (**B**), and hydroxycinnamic acids (**C**). Also see *UNIT I1.3*.

acids. Most hydroxycinnamic acids are rarely found naturally in the free state, but occur partly as glucose esters, and most frequently as quinic acid esters (Herrmann, 1989; Möller and Herrmann, 1983). Chlorogenic acid, commonly distributed in fruits and vegetables, consists of a combination of quinic and caffeic acids.

Flavonoids have the common structure of diphenylpropane (C_6-C_3-C_6), consisting of two aromatic rings joined together by three carbons that are usually formed into an oxygenated heterocyclic ring (Figure I1.2.4). The structures and numbering systems of the various classes of flavonoids are shown in *UNIT I1.3* (see Figures I1.3.4 to I1.3.9). The oxidative state of this three-carbon chain determines the various flavonoids' classes. Flavonoids include anthocyanins, flavanols (catechins), flavonols, flavones, isoflavones, flavanones, and their derivatives. The most common flavonoids are flavones, flavonols, and their derivatives. The anthocyanins are water-soluble pigments responsible for the red, purple, and blue colors in fruits and flowers. Anthoxanthins refer to flavones, flavanones, flavonols, and isoflavones, which are colorless or white to yellow molecules (King and Young, 1999). Isoflavones, structural isomers to flavonoids, are found mainly in soy and soy-based products (Choi et al., 2000). Most flavonoids, except flavan-3-ols, are usually glycosylated, with some of the phenolic hydroxyl groups linked to sugar residues such as glucose, rhamnose, galactose, and arabinose (Lee, 2000).

Tannins are polyphenolic compounds of high molecular weight that occur naturally and react with proteins. They can form insoluble complexes with proteins, which are responsible for the taste known as astringency that is caused by precipitation of mouth proteins. Tannins are typically classified into three groups: (1) the condensed tannins, (2) the hydrolyzable tannins, and (3) the phlorotannins (Figure I1.3.10). Condensed tannins (also referred to as procyanidins or proanthocyanidins) are oligomers or polymers of two or more catechins or epicatechins. Hydrolyzable tannins are polymers of gallic or ellagic acid, which are easily hydrolyzed by acid, alkali, or enzymes. Phlorotannins are made up of phloroglucinol subunits. Tannins having redox potentials similar to those of related simple phenolic compounds are 15 to 30 times more effective than Trolox at scavenging peroxy radicals (Hagerman et al., 1998).

Extraction of polyphenolics

The extraction is the main step in the recovery and isolation of polyphenolics and the evaluation of individual and total polyphenolics from various plant-based materials. The objective of solvent extraction is to remove all polyphenolics from the solid plant material by transferring them into a liquid phase. Polyphenolics are often most soluble in solvents less polar than water. An effective extraction of plant material depends on a proper solvent selection, elevated temperatures, and mechanical agitation to maximize polyphenolics recovery. Some polyphenolics are subject to degradation during sample preparation and extraction due to light and oxidation. Using ascorbic acid (Lee and Jaworski, 1987), sodium metabisulfite (Donovan et al., 1998), or nitrogen gas purging (Kim et al., 2002) during sample preparation will prevent oxidation of polyphenolics. It is recommended that samples be analyzed within 1 day of extraction.

Choice of solvents

The most common solvents used for the extraction of polyphenolics from plant material are methanol, ethanol, acetone, ethyl acetate, and their aqueous solvents. Aqueous methanol is a popular choice of solvent because it is efficient, has a high boiling point, and is eco-

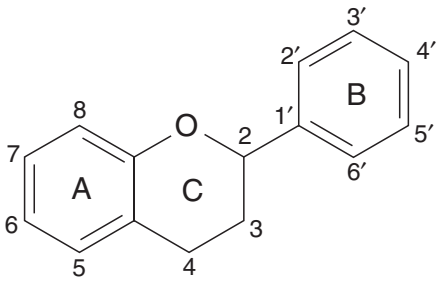

Figure I1.2.4 Structure and numbering system of flavonoids. Also see *UNIT I1.3*.

nomical. Methanol has been successfully used in extracting polyphenolics from various vegetables and fruits (Hertog et al., 1992a), such as apples (Oleszek et al., 1988), grapes (Lee and Jaworski, 1990), onions (Park and Lee, 1996), prunes and prune juice (Donovan et al., 1998), and banana bracts (Pazmiño-Durán et al., 2001). Acetone has been used to extract polyphenolics from apples (Eberhardt et al., 2000), prunes (Nakatani et al., 2000), and blackberries (Stintzing et al., 2002). Acetone extraction of anthocyanins is presented in *UNIT F1.1*. Ethanol has been used to extract polyphenolics from apples (Coseteng and Lee, 1987), grapes (Jaworski and Lee, 1987), and buckwheats (Kreft et al., 1999). Polyphenolics in wine and olive oil residues have been efficiently extracted with ethyl acetate (Lesage-Meessen et al., 2001; Salagoïty-Auguste and Bertrand, 1984). Polyphenolics such as catechin, epicatechin, and procyanidins are traditionally extracted from teas by brewing with hot water (Wang et al., 2000a,b).

Rutin extraction from buckwheat was optimally performed using between 50% and 60% ethanol with a 3-hr maceration (Kreft et al., 1999). Repeating the extraction two times was sufficient to extract most of the rutin. Lie et al. (2000) demonstrated the optimization of influencing factors such as extraction temperatures, extraction times, and solvent concentrations in a conventional extraction of dry powdered material of *Hypericum perforatum*. Applied extraction conditions caused variations to favor different components in the mixture. Higher extraction efficiency was achieved with moderately polar solvents to extract flavonoids such as rutin, isoquercitrin, and quercetin. The optimum extraction conditions for dry *Hypericum perforatum* leaf powder were determined as 44% to 69% ethanol in acetone with a 5.3- to 5.9-hr maceration at 55°C.

In order to extract anthocyanins in plant material, Basic Protocol 1 and Alternate Protocol 1 can be used if the extraction system is applied at a low pH (<3.0). The acidic environment is generally provided by the addition of hydrochloric acid or other organic acids to the solvent (Donner et al., 1997; Chandra et al., 2001; Pazmiño-Durán et al., 2001). Extraction of anthocyanins in an acidic environment is desirable because the red flavylium cation is then more stable. During the evaporation process, however, anthocyanins in extracts containing weak organic or mineral acids may be subject to acid hydrolysis of labile acyl groups and substituted sugars. To avoid such acid hydrolysis, anthocyanins can be extracted only by solvents without the addition of acids. For more detailed information, refer to *UNIT F1.1*, which extensively discusses and reviews anthocyanin extraction.

Sonication and homogenization

The traditional extraction method usually requires a long extraction time (Nakatani et al., 2000). Recently, ultrasound was applied effectively and rapidly to extract polyphenolics from plant material. The low frequency of ultrasound (in the kHz range) has the advantage of aiding extraction with significantly reduced extraction times and enhanced extraction yield. The efficient extraction of polyphenolics by ultrasound is ascribed to the cavitational phenomenon, which is the rapid formation and collapse of countless microscopic bubbles in a solvent produced by the alternating low- and high-pressure waves generated by ultrasonic sound. Bubbles filled with solvent vapor are produced at low pressure and then are compressed and finally implode at high pressure, leading to a powerful shock wave and enhanced mixing in the solvent extraction system (Paniwnyk et al., 2001). High-power ultrasound improves solvent extraction from plant material mainly due to its mechanical effects (Mason et al., 1996). These effects via cavitational collapse of bubbles enhance mass transfer rate and solvent penetration into cellular materials. In addition, the disruption or damage of biological cell walls by ultrasound results in facilitated release of the intracellular contents.

The extraction with methanol using a sonicator under strict exclusion of light at a low temperature is rapid and efficient, with >99% of the major constituents extracted from St. John's Wort in two 30-min steps of sonication (Li and Fitzloff, 2001). Methanolic extraction with ultrasound gave a significant increase of maximum yield and reduction of extraction time compared to conventional reflux extraction (Paniwnyk et al., 2001). In any extraction procedure, the solubility of the extracted components at a given extraction temperature should be considered when selecting the solvent. Ultrasonic extraction (three times for 1.5 hr each time) with unregulated temperature was comparable to water-bath extraction (three times for 5.6 hr each time at 55°C). The former showed an advantage of being less time consuming even with the drawback of the fluctuating temperature of an ultrasonic bath (Liu et al., 2000). Ultrasonic extraction with aqueous methanol to extract phenolic phytochemicals from ap-

ples under a nitrogen environment was effectively employed (Kim et al., 2002). Ultrasound-assisted extraction used at uniformly low temperatures can avoid thermal degradation and reduction of cavitational effects. It is recommended that ultrasound-assisted extraction also be used with additional agitation or shaking to avoid standing waves or the formation of solid-free regions for the preferential flow of the ultrasonic waves (Vinatoru et al., 1997).

Using both blender and homogenizer (see Alternate Protocol 1) has the advantage of immediate maceration in absolute methanol after briefly washing fresh plant material. This enhances the prevention of oxidative degradation during sample preparation for fresh plant-based materials. In addition, reducing agents such as ascorbic acid and sodium metabisulfite should be incorporated into the extraction procedure to prevent oxidation and browning reactions. Absolute methanol is used instead of aqueous methanol due to the water content of fresh, raw plant material.

Isolation/purification

Since considerable amounts of potential interfering materials can be extracted along with the polyphenolics, an isolation/purification step is often required to eliminate components that may interfere with analysis. The fractionation techniques presented in Basic Protocol 2 and Alternate Protocol 2, using solid-phase extraction to minimize the effects of sample preparation/cleanup on the integrity of the extract, will make possible the identification and quantification of individual polyphenolics by HPLC (*UNIT I1.3*), MS, and NMR.

Solid-phase extraction using a small, disposable Sep-Pak C18 cartridge has been popularized as the preferred method for cleaning and fractionating phenolic acids and flavonoids in order to remove interfering substances from crude polyphenolics extracts. The C18 solid-phase extraction technique has been used to isolate polyphenolics from vegetables, grapes, cherries, and apples. One of the major problems involved in the separation of polyphenolics is their similarity in chemical characteristics (Jaworski and Lee, 1987). A simple fractionation method was developed by Oszmianski and Lee (1990b) using a preconditioned Sep-Pak C18 cartridge to separate non-anthocyanin polyphenolics from anthocyanins for the successful analysis of non-anthocyanin polyphenolics. In this method, polyphenolics were absorbed onto the solid phase. Sugars, organic acids, and other polar components in the loaded cartridge were eluted with acidified water and the cartridge was dried by means of a continual current of nitrogen gas for several minutes. Polyphenolics other than anthocyanins were eluted from the cartridge using ethyl acetate. Anthocyanins still bound to the solid phase were eluted with acidified methanol (0.1% [v/v] HCl/methanol). A crucial step in this fractionation process is the removal of water from the cartridge using nitrogen gas. The recoveries from red grapes is as follows: ~100% for caffeoyl tartrate, 86% to 91% for procyanidin B3, 77% for rutin, and 98% to 99% for anthocyanins (Oszmianski and Lee, 1990b).

Phenolic acids are completely ionized at pH 7 and completely un-ionized at pH 2 (Salagoïty-Auguste and Bertrand, 1984). This characteristic allows for solid-phase extraction of neutral polyphenolics (catechins, procyanidins, flavonols, isoflavonoids) at pH 7 and acidic phenolics (caffeoyl tartrate, *p*-coumaroyl tartrate) at pH 2. Jaworski and Lee (1987) recovered >90% of acidic phenolics, catechin, and epicatechin, 104% of procyanidin B2, and 118% of procyanidin B3 by passing deproteinated grape juice through a Sep-Pak C18 cartridge. [Recoveries >100% might commonly take place due to experimental error (5% to 10%) from each experiment.] This simple, easy-to-use fractionation method also improved the resolution of many polyphenolic peaks by HPLC analysis. It was thus suggested that it facilitates the collection of unknown peaks individually, making possible their analysis and identification. However, the degradation of sample integrity can result from changes in pH, which may result in hydrolysis of acyl and glycosidic linkages, oxidative reaction of polyphenolics, and isomerization of cinnamic acids or esters (Roggero et al., 1990).

In a polyphenolic extract, anthocyanins can interfere with other polyphenolics such as procyanidins during HPLC analysis and hence should be removed prior to analysis. Anthocyanins from crude polyphenolic extracts can be removed as described in Basic Protocol 2. The ethyl acetate used for elution of phenolic compounds other than anthocyanins is removed using a rotary evaporator at 20°C. The non-anthocyanin polyphenolics are dissolved in deionized distilled water and the pH is adjusted to 7.0 with NaOH as described in Alternate Protocol 2 or the method developed by Oszmianski and Lee (1990a). In the latter method, polyphenolics were fractionated into three groups: neutral fraction A (flavanols and other polar phenolics), neutral fraction B (flavonols), and acidic phenolics. Polyphenolic extracts were adjusted to pH 7.0 with NaOH

and passed through two C18 Sep-Pak cartridges connected together and preconditioned with 5 ml of methanol and 5 ml of deionized distilled water. The acidic fraction was washed with 5 ml of deionized distilled water, and 5 ml of 0.01 N HCl. Neutral fraction A absorbed on the solid phase was eluted with 5 ml of 10% acetonitrile solution acidified to pH 2.0. To elute neutral fraction B, 5 ml of 40% acetonitrile was used. The acidic fraction was adjusted to pH 2.0 with HCl and passed through a C18 cartridge preconditioned with 5 ml methanol and 5 ml of 0.01 N HCl. The absorbed phenolics were eluted with 5 ml of 40% acetonitrile. Prior to solid-phase extraction, the polyphenolic extract was subjected to phase separation with hexane to remove carotenoids and other nonpolar compounds.

Besides solid-phase extraction, column chromatography is also often used for cleanup and purification of polyphenolics from plant material. Ionic adsorbants (polyvinylpyrrolidone or PVP, polyamides, and Sephadex LH-20) and Amberlite XAD-2 resin have been used to isolate and purify polyphenolics from crude extracts. For the separation of polyphenolics from plant material, column chromatography using Sephadex LH-20, a gel-filtration matrix, is often used with various eluting solvents (Park and Lee, 1996). The most widely used solvents for column chromatography are aqueous methanol and aqueous ethanol.

Critical Parameters and Troubleshooting

The sonication time, repetitions of extraction, solvent polarity, and solvent selection may depend on the particular plant material. Before using Basic Protocol 1, the optimization of these parameters must be determined in order to efficiently extract polyphenolics from plant materials. The use of finely ground samples enhances polyphenolics recovery due to the increased surface area and easy disruption of biological cellular walls. During extraction, a continual supply of nitrogen gas should be applied to the extraction vessel to provide a nitrogen environment, thus avoiding any possible oxidative degradation.

For an accurate quantification of phenolic compounds extracted from plant material, the starting plant material should be washed, dried, and weighed prior to extraction, and the amount of the final extract should be recorded. The extracted polyphenolics are generally diluted and then concentrated by evaporation of solvents. Evaporation should be performed at 30° to 40°C under reduced pressure. When extracting anthocyanins using acidic conditions for their stability (see Background Information), it should be kept in mind that acid hydrolysis of polyphenolics may take place during the concentration process. The final extract can be quantified based on gallic acid or catechin equivalents using spectrophotometric measurement before HPLC (Singleton and Rossi, 1965).

Ultrasound tends to increase the temperature of the ultrasonic water bath, which may cause pigment degradation in the extract. Without the use of a temperature control device, a temperature increase from 23° to 65°C was reported during 90 min of ultrasonication (Liu et al., 2000). The surrounding temperature and air flow inside a fume hood may also affect the temperature in the ultrasonic bath. The temperature of an ultrasonic water bath should be controlled to prevent degrading reactions during extraction at room temperature or at lower temperatures. This can be achieved by using a continuous cooling system or by periodically adding ice to the water bath. In addition to the ultrasonic bath, agitation or shaking is desirable to avoid standing waves or the formation of solid-free regions for the preferential flow of the ultrasonic waves.

After filtering the mixture of sample and organic solvent, the hose connected from the water aspirator to the flask under the Buchner funnel should first be carefully disconnected before shutting off the water source to prevent possible backflow of tap water into the flask, which would contaminate the sample. Prior to evaporation, the solvent waste reservoir should be empty, clean, and dry. Evaporation should be started slowly at reduced pressure to avoid the boiling over of solvent and samples. If by chance the polyphenolic extract boils over into the waste reservoir, immediately release the applied vacuum and transfer the extract back to the original boiling flask. If this is not satisfactory and contamination is a possibility, extraction should be performed again with a new sample.

The final extract after evaporation may be partitioned with hexane in a separatory funnel to remove chlorophyll, lipids, carotenoids, and other fat-soluble materials (e.g., in avocado, olives, seeds), if considerable amounts of these compounds are present. However, possible loss of lipophilic polyphenolics should be checked. Performing the liquid-liquid extraction after the evaporation of solvent may reduce the amount of hexane required. The extract should be flushed with nitrogen gas and stored in the

dark at 4°C to avoid possible loss of sample due to oxidation and other degrading reactions. If the analysis is prolonged, the extract should be stored at −18°C or below in a freezer-resistant container. Flavones and flavonols in methanol proved to be stable for >3 months at 4°C (Hertog et al., 1992b). Extracts at −18°C might be stable at least 3 months.

C18 solid-phase extraction is used to fractionate polyphenolics for their identification and characterization. This technique can eliminate interfering chemicals from crude extracts and produce desirable results for HPLC or other analytical procedures. To obtain a sufficient volume for all analyses, several separations by solid-phase extraction may be performed. The individual fractions need to be combined and dissolved in solvents appropriate for HPLC analysis. In Basic Protocol 2, the application of a current of nitrogen gas for the removal of water from the C18 cartridge is an important step in the selective fractionation of polyphenolics into non-anthocyanin and anthocyanin fractions. After the collection of non-anthocyanin polyphenolics, no additional work is necessary to elute anthocyanins bound to the C18 solid phase if anthocyanins are not to be determined.

Anticipated Results

Ultrasound-assisted extraction provides efficient extraction in a shorter processing time than is needed for conventional extraction. The aid of ultrasound will result in a higher extraction yield and reduce solvent consumption. The extract may exhibit a wide range of colors (pale yellow, brown, red). For anthocyanin extraction in an acidic environment, the extract will be deep red, pink, or purple. The extract may contain considerable amounts of lipophilic compounds (e.g., chlorophyll, carotenoids, lipids). Prior to solid-phase extraction, those compounds can be eliminated from the extracts using liquid-liquid extraction.

Time Considerations

The ultrasound-assisted extraction of freeze-dried plant-based materials normally takes ~2 hr. If the extract is evaporated to dryness, a total of ~3 hr is necessary. As an additional sample preparation step, 2 to 4 days should be allotted for freeze-drying fresh plant materials, depending on the quantity of the material. Homogenizer-assisted extraction of fresh fruit takes <4 hr.

Isolation/purification using solid-phase extraction is a fast step, generally needing ≤3 hr. Prior to this solid-phase extraction, if it is necessary to remove chlorophyll, carotenoids, and other lipophilic compounds from the final extracts, liquid-liquid partitioning with hexane takes an additional 90 min, depending on the particular plant material.

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Key References

Jaworski and Lee, 1987. See above.

An improved analytical method for the fractionation of polyphenolics into neutral and acidic groups by passing deproteinated grape juice through a preconditioned C18 Sep-Pak cartridge.

Oszmianski and Lee, 1990b. See above.

A method for fractionation of polyphenolics into non-anthocyanin and anthocyanin fractions from red grapes by solid-phase extraction using disposable C18 cartridges.

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HPLC Separation of Polyphenolics

The polyphenolics, ubiquitous phytochemicals in the plant kingdom, are important aromatic secondary metabolites of plants. Diverse combinations of polyphenolics are found in plant-based materials. Polyphenolics are important because they are responsible for the color and flavor of fresh and processed products. Some have strong antioxidant and anticancer activities. They are routinely consumed in the human diet in significant quantities.

In this unit, methods for reversed-phase high-performance liquid chromatography (HPLC) are described for the analysis of polyphenolics. HPLC analysis can be employed in an easy and fast manner to obtain an accurate elucidation and quantification of individual polyphenolic compounds found in plant-based materials. The separation of each polyphenolic is based on the polarity differences among polyphenolics with structural similarities and uses various combinations of mobile and stationary phases.

The Basic Protocol describes the reversed-phase HPLC analysis of polyphenolic compounds isolated into nonanthocyanin and anthocyanin fractions by solid-phase extraction. The Alternate Protocol describes the HPLC separation of acidic and neutral polyphenolic fractions. Fractionated samples are used because significant amounts of interfering compounds are extracted along with polyphenolics from plant materials. Solid-phase extraction with C18 Sep-Pak cartridges (*UNIT I1.2*) is used to selectively eliminate undesired components from crude extracts, and may minimize the effects of sample cleanup or preparation on the integrity of polyphenolics. The isolation and purification step using solid-phase extraction of polyphenolics will make possible the efficient analysis of individual polyphenolics by reversed-phase HPLC.

NOTE: Deionized and distilled or HPLC-grade water should be used in these protocols. Reagents (solvetns) should also be HPLC grade.

REVERSED-PHASE HPLC ANALYSIS OF POLYPHENOLICS SEPARATED INTO NONANTHOCYANIN AND ANTHOCYANIN FRACTIONS

BASIC PROTOCOL

In this protocol, polyphenolics isolated as nonanthocyanin and anthocyanin fractions after a sample cleanup are analyzed by reversed-phase HPLC in order to obtain an accurate measurement of individual polyphenolic constituents.

Materials

Crude polyphenolic sample (*UNIT I1.2*)
Methanol
Acidified aqueous methanol or acidified water (0.1% [v/v] HCl)
Mobile phases:
- 50 mM $(NH_4)H_2PO_4$, pH 2.6 (pH adjusted with orthophosphoric acid)
- 80:20 (v/v) acetonitrile/50 mM $(NH_4)H_2PO_4$, pH 2.6
- 200 mM H_3PO_4, pH 1.5 (pH adjusted with ammonium hydroxide)

Polyphenolic standards (see Table I1.3.1 for suppliers)

0.45-µm poly(tetrafluoroethylene) (PTFE) syringe-tip filters
High-performance liquid chromatography (HPLC) system equipped with:
- Quaternary pump
- Diode array detector
- Vacuum degasser

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Table I1.3.1 Elution Order of Polyphenolic Compounds Separated by HPLC of Nonanthocyanin and Anthocyanin Fractions[a]

Compound[b]	Retention time (min)
280 nm:	
Gallic acid	8.739 ± 0.155
Protocatechuic acid	12.948 ± 0.161
Epigallocatechin	15.637 ± 0.215
Catechin	17.998 ± 0.209
Vanillic acid	20.247 ± 0.229
Syringic acid	21.612 ± 0.283
Epicatechin	24.697 ± 0.391
Epigallocatechin gallate	25.710 ± 0.285
Epicatechin gallate	33.209 ± 0.110
Naringin	34.626 ± 0.039
320 nm:	
Chlorogenic acid	17.594 ± 0.175
Caffeic acid	21.553 ± 0.331
p-Coumaric acid	30.315 ± 0.281
Sinapic acid	32.266 ± 0.108
Ferulic acid	32.301 ± 0.167
Resveratrol	38.022 ± 0.121
Cinnamic acid	40.953 ± 0.088
370 nm:	
Rutin	32.226 ± 0.149
Rhoifolin	34.395 ± 0.040
Quercitrin	34.668 ± 0.071
Myricetin	36.478 ± 0.079
Luteolin	39.335 ± 0.097
Quercetin	39.752 ± 0.115
Apigenin	41.913 ± 0.048
Kaempferol	42.548 ± 0.194
520 nm:	
Kuromanin	19.525 ± 0.307
Malvin	19.855 ± 0.519
Keracyanin	20.879 ± 0.139
Delphinidin	26.590 ± 0.284
Oenin	28.130 ± 0.059
Cyanidin	30.523 ± 0.085
Pelargonidin	32.626 ± 0.041
Peonidin	33.160 ± 0.165
Malvidin	33.213 ± 0.056

[a]Retention time will depend on the specific column used. All results are expressed as mean ± SD for at least twenty replications, and were obtained on a Symmetry (Waters Chromatography) analytical column.

[b]Sigma is an appropriate supplier of all 280-, 320-, and 370-nm compounds; 520-nm compounds can be purchased from Extrasynthese. Other suppliers include Indo Fine Chemical and Polyphenols AS.

5-µm × 250-mm × 4.6-mm C18 reversed-phase column (e.g., Symmetry; Waters Chromatography)
Guard column (e.g., Symmetry Sentry; Waters Chromatography)
1- to 100-µl sample injection loop (20-µl loop is recommended)

Additional reagents and equipment for fractionating crude polyphenolics by solid-phase extraction into anthocyanin and nonanthocyanin fractions (*UNIT I1.2*)

1. Fractionate a crude polyphenolic sample into anthocyanin and nonanthocyanin fractions by solid-phase extraction as described in *UNIT I1.2*.

   For quantitative analysis by HPLC, accurate weights of the raw materials used and final volumes of extracts should be recorded. The sample volume applied to the disposable C18 cartridge, the final sample volume after evaporation, and the dissolving solvent volume must also be known.

   This fractionation step may be optional. Some samples can be directly analyzed by HPLC after filtration (step 2) without solid-phase extraction. Anthocyanins that can be detected at 280 nm can interfere with the separation of some polyphenolics. If the analyst is interested in nonanthocyanin polyphenolics, and especially if plant materials containing high levels of anthocyanins are being analyzed, this fractionation technique should be utilized.

2. Dilute anthocyanin and nonanthocyanin fractions 1:1 (v/v) with methanol and filter each through a 0.45-µm PTFE syringe-tip filter. For the anthocyanin fraction, use acidified aqueous methanol or acidified water as the solvent.

   See UNIT F1.3 for more detailed information about anthocyanin separation by HPLC.

3. Set up an HPLC system with a 5-µm × 250-mm × 4.6-mm C18 reversed-phase column and guard column. Set flow rate to 1.0 ml/min at constant room temperature (e.g., 23°C).

   Installation of a guard column having the same packing material as the main column lengthens the life of the often expensive analytical column.

   The instrument room should be maintained at constant temperature for greater reproducibility during HPLC analysis. The operating temperature and flow rate may be altered depending on the particular HPLC system.

4. Set detector at 280 nm for catechins (flavan-3-ols), naringin, and benzoic acid derivatives; 320 nm for chlorogenic acid, resveratrol, and hydroxycinnamic acids; 370 nm for flavones and flavonols; and 520 nm for anthocyanins and anthocyanidins (see Table I1.3.1).

   Detection of the nonanthocyanin fraction at 280, 320, and 370 nm can be done simultaneously. For direct injection after filtration without fractionation, or for analysis of combined nonanthocyanin and anthocyanin fractions, all four wavelengths can be used simultaneously.

5. Load a 20-µl fractionated polyphenolic sample into the HPLC system.

   Although the use of a 20-µl injection loop is recommended, the injection volume applied to the HPLC system may depend on the samples used. Injection volumes between 1 and 100 µl are generally used (Merken and Beecher, 2000).

6. Run mobile phases as described in Table I1.3.2.

   To enhance peak resolution, the user may modify the solvent gradient. The linear-gradient mobile phases used here are modified from Lamuela-Raventós and Waterhouse (1994).

7. Use the polyphenolic standards to generate characteristic UV-Vis spectra and retention times and identify individual polyphenolics in the sample.

Table I1.3.2 Solvent Gradient for Reversed-Phase HPLC Analysis of Polyphenolics[a]

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
4	92	8	0
10	0	14	86
22.5	0	16.5	83.5
27.5	0	25	75
50	0	80	20
55	100	0	0
60	100	0	0

[a]Solvent A, 50 mM $(NH_4)H_2PO_4$, pH 2.6; solvent B, 80:20 (v/v) acetonitrile/solvent A; solvent C, 200 mM H_3PO_4, pH 1.5.

The elution order of commercially available standards is presented in Table I1.3.1. Retention times of the peaks will depend on the specific column used.

8. Analyze polyphenolic standards at a minimum of three concentrations to generate calibration curves. Analyze data and calculate the quantity of each polyphenolic compound.

   A range of 0 to 10 ppm should be used for the concentrations of the standards, which should produce a linear relationship.

ALTERNATE PROTOCOL

REVERSED-PHASE HPLC ANALYSIS OF POLYPHENOLICS SEPARATED INTO NEUTRAL AND ACIDIC FRACTIONS

Phenolic acids are ionized at pH 7.0 and are un-ionized at pH 2.0. This property allows for solid-phase extraction of neutral polyphenolics at pH 7.0 and acidic polyphenolics at pH 2.0 to prevent interference. In this protocol, polyphenolics isolated as neutral and acidic fractions using pH adjustments are analyzed by reversed-phase HPLC in a thermostatically controlled environment.

Additional Materials *(also see Basic Protocol)*

Mobile phases:
5:95 (v/v) acetic acid/water (for acidic fractions)
5:95 (v/v) acetic acid/water and 40:60 (v/v) acetonitrile/water (for neutral fractions)
Binary pump
8 × 100–mm C18 Radial-Pak column (Waters Chromatography) and guard column

Additional reagents and equipment for fractionating crude polyphenolics into acidic and neutral fractions (*UNIT I1.2*)

1. Fractionate a crude polyphenolic sample into acidic and neutral fractions as described in *UNIT I1.2*.

   For quantitative analysis by HPLC, accurate weights of raw materials used and final volumes of extracts should be recorded. The sample volume applied to the disposable C18 cartridge, the final sample volume after evaporation, and the dissolving solvent volume must also be known.

   This particular fractionation step may be optional. Some samples can be directly injected after filtration (step 2) without solid-phase extraction. This technique, however, will improve the resolution of many of the HPLC polyphenolic peaks and will allow their analysis and identification.

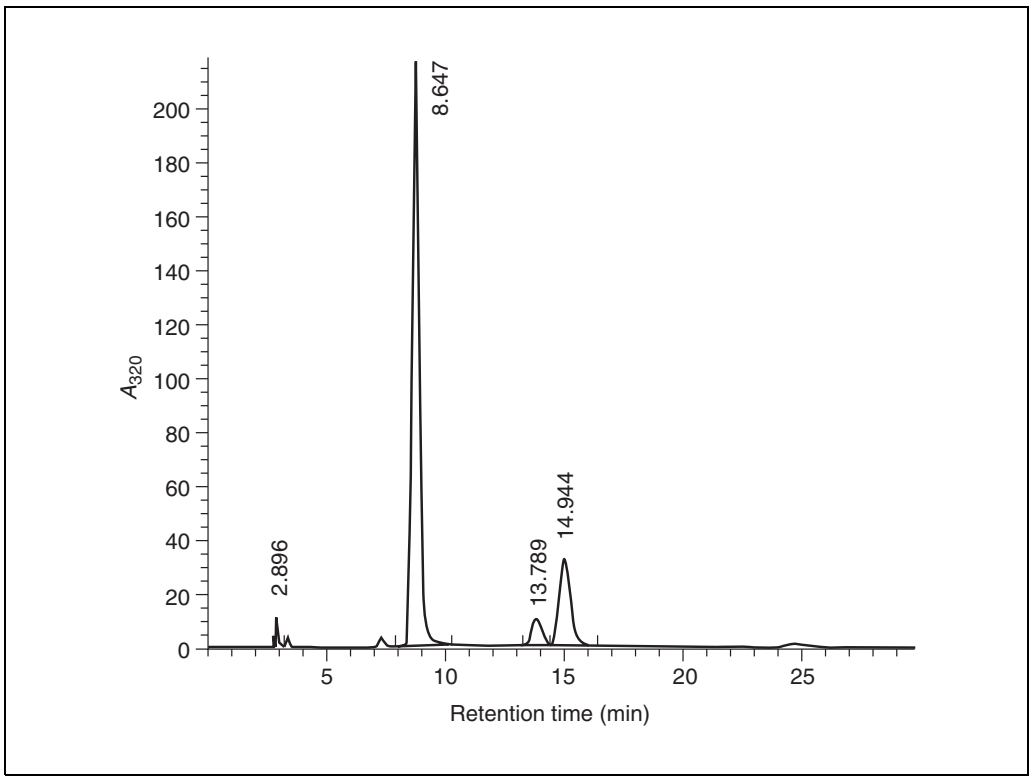


Figure I1.3.1 HPLC chromatogram of acidic polyphenolics isolated from Niagara grapes and detected at 320 nm. Retention time: 8.647 min, *trans*-caftaric acid; 13.789 min, *cis*-coutaric acid; 14.944, *trans*-coutaric acid. Reproduced from Lee and Jaworski (1987) with permission from the American Society for Enology and Viticulture.

2. Dilute acidic and neutral fractions 1:1 (v/v) with methanol and filter through a 0.45-µm PTFE syringe-tip filter.
3. Set up an HPLC system (see Basic Protocol, steps 3 and 4), but use a binary pump and an 8 × 100–mm C18 Radial-Pak column, and set the detector at 320 nm for acidic polyphenolics or 280 nm for neutral polyphenolics. Load column as in step 5 of the Basic Protocol.

4a. *For acidic polyphenolics:* Elute isocratically with 5:95 acetic acid/water for 30 min.

Retention times of the peaks are subject to the particular type of column. The acidic fraction from solid-phase extraction consists of phenolic acids such as cis-coutaric, trans-coutaric, and trans-caftaric acids. Isocratic elution is suitable because of the limited number of compounds found in the acidic fraction. Analysis of the acidic fraction is completed within 30 min. See Figure I1.3.1 for an HPLC chromatogram of the acidic polyphenolics isolated from Niagara grapes.

Table I1.3.3 Solvent Gradient for Reversed-Phase HPLC Analysis of Neutral Polyphenolics[a]

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
1	100	0
50	0	100
55	100	0
60	100	0

[a]Solvent A, 5:95 (v/v) acetic acid/water; solvent B, 40:60 (v/v) acetonitrile/water.

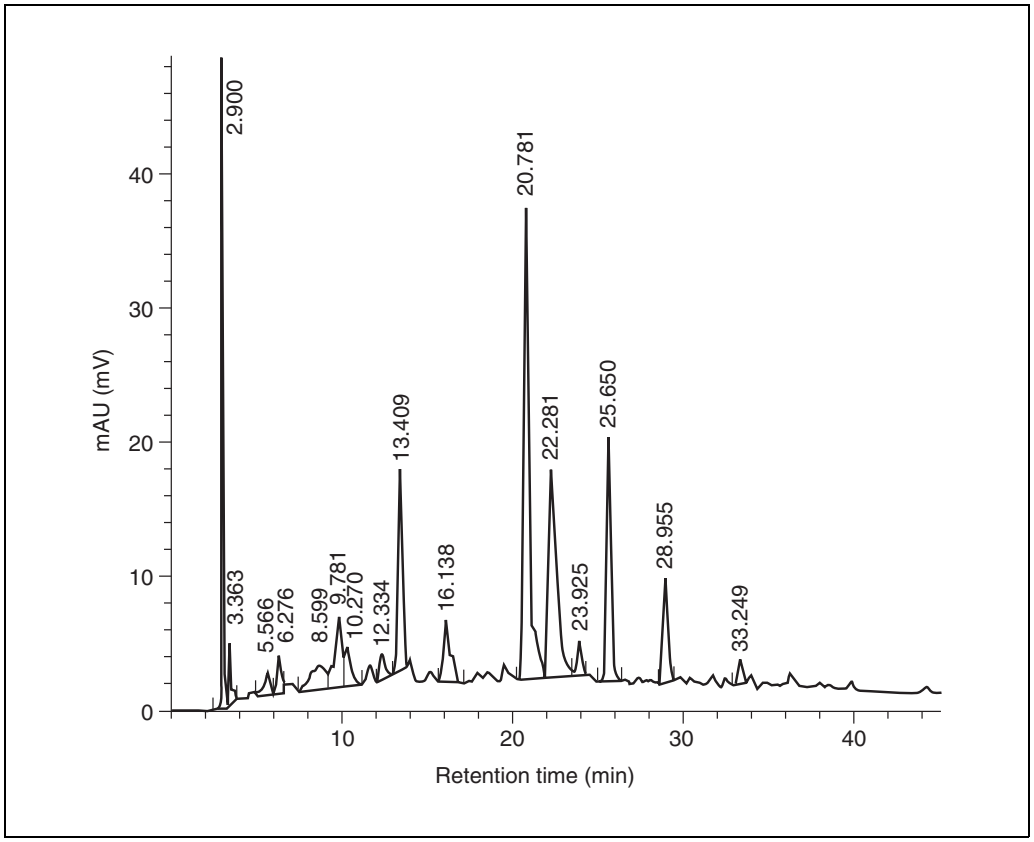


Figure I1.3.2 HPLC chromatogram of neutral polyphenolics found in Niagara grapes detected at 280 nm. Retention time: 8.599 min, procyanidin B3; 9.781 min, procyanidin B1; 13.409 min, catechin; 16.138 min, procyanidin B2; 20.781 min, epicatechin; 22.281 min, catechin-catechin-gallate; 23.925 min, catechin-catechin-gallate isomer; 28.955 min, catechin-gallate. AU, absorbance units. Reproduced from Lee and Jaworski (1987) with permission from the American Society for Enology and Viticulture.

4b. *For neutral polyphenolics:* Elute using 5:95 acetic acid/water and 40:60 acetonitrile/water over 60 min, as presented in Table I1.3.3.

Retention times of the peaks are subject to the particular type of column. For enhanced resolution of the peaks, the solvent gradient may be altered. See Figure I1.3.2 for a chromatogram of neutral polyphenolics found in Niagara grapes.

5. Analyze polyphenolic standards and quantify polyphenolics in the sample as described (see Basic Protocol, steps 7 and 8).

COMMENTARY

Background Information

Polyphenolic phytochemicals are ubiquitous in the plant kingdom. These important aromatic secondary metabolites of plants are consumed in significant amounts in daily life. Their occurrence among plant-based materials is frequently varied. They contribute to sensory qualities such as color, flavor, and taste of plant-based materials. The composition of polyphenolic phytochemicals is influenced by maturity, cultivar (Lee and Jaworski, 1987), cultural practices, geographic origin, climatic conditions, storage conditions, and processing procedures (Spanos and Wrolstad, 1990). Some phytochemicals are known as nutraceuticals, which provide health benefits because of their biological activities (Dillard and German, 2000). Research on phytochemicals has been driven in recent years by their beneficial health effects, including antioxidant, anticarcinogenic, and antimutagenic activities (Huang and Ferraro, 1992) and their ability to reduce the risk of coronary heart disease (Hertog et al., 1993).

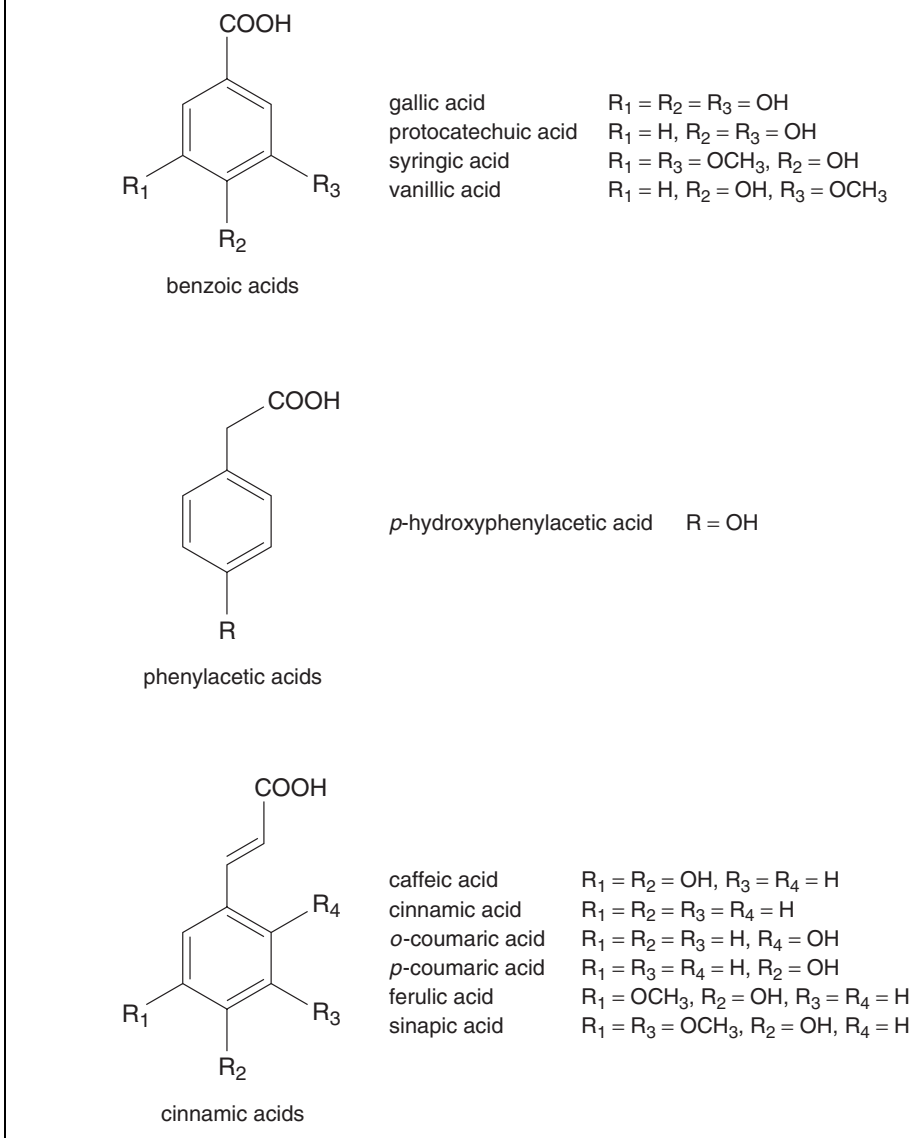


Figure I1.3.3 Structure of phenolic acids.

Polyphenolic phytochemicals are classified into three major groups: phenolic acids, flavonoids, and tannins. Phenolic acids include hydroxybenzoic, hydroxyphenylacetic, and hydroxycinnamic acids (Figure I1.3.3). Hydroxycinnamic acids are the most widely distributed of the phenolic acids in plant tissues. The important hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids. Most hydroxycinnamic acids are rarely encountered in the free state in nature. They occur as glucose esters and, more frequently, as quinic acid esters (Herrmann, 1989). Phenolic acids are usually detected at wavelengths between 210 and 320 nm. In general, the polarity of phenolic acids is increased mainly by the hydroxyl group at the 4 position, followed by those at the 3 and 2 positions. Methoxyl and acrylic groups substituted into the aromatic ring reduce polarity and increase the retention times (Torres et al., 1987). When using the Basic Protocol, the elution order for benzoic acids is gallic acid, protocatechuic acid, vanillic acid, and syringic acid (Rodríguez-Delgado et al., 2001; Table I1.3.1). The elution order for hydroxycinnamic acids is caffeic acid, *p*-coumaric acid, sinapic acid, ferulic acid, and cinnamic acid (Table I1.3.1).

The flavonoids are the largest and most important group of plant phenolics. They have the common structure of diphenylpropane ($C_6C_3C_6$; Figure I1.3.4A). The flavonoids are

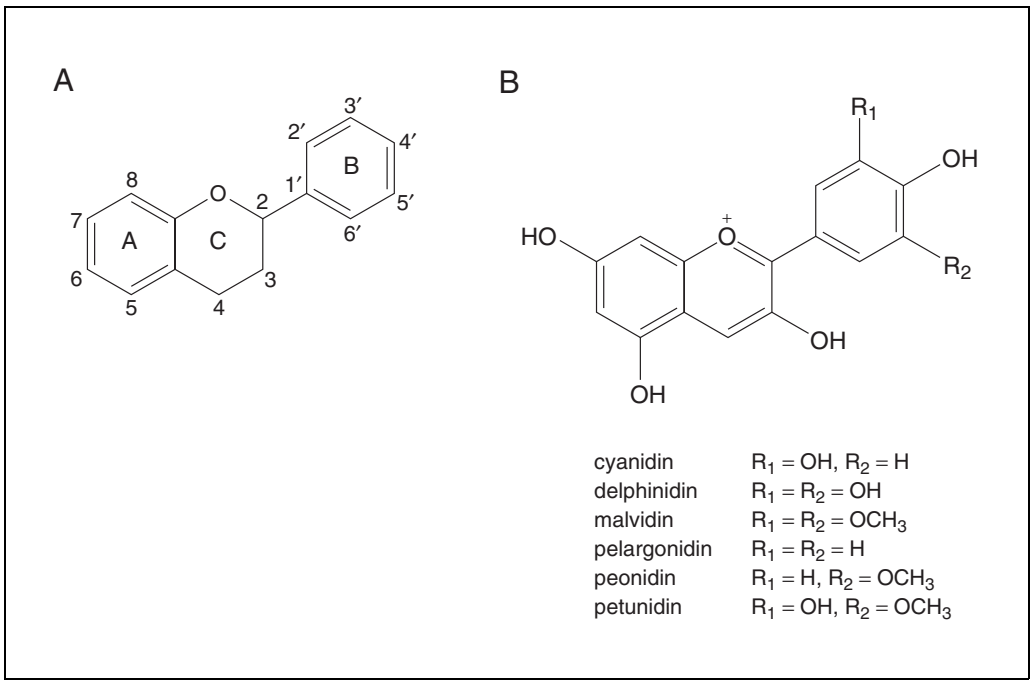


Figure I1.3.4 (**A**) Generic structure and numbering convention of flavonoids, which have a common diphenylpropane ($C_6C_3C_6$) structure. (**B**) Structure of anthocyanidins.

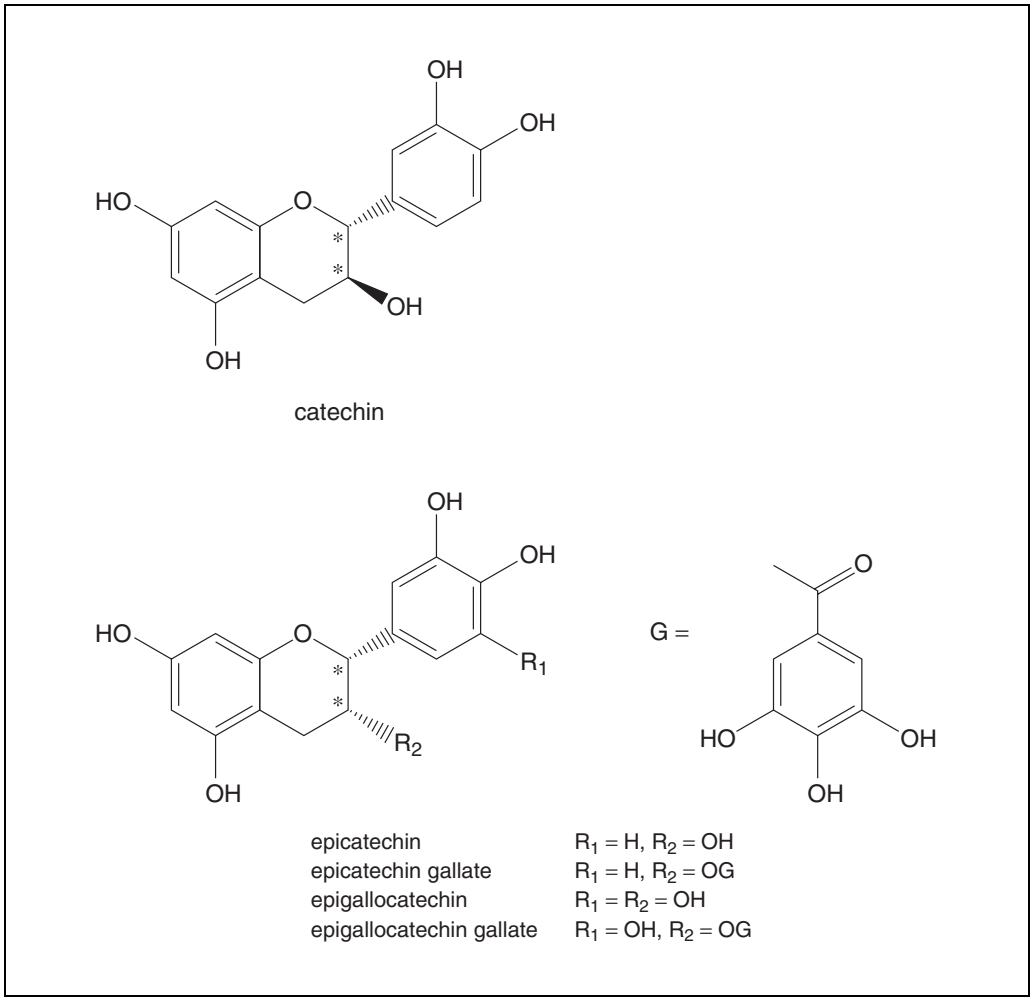
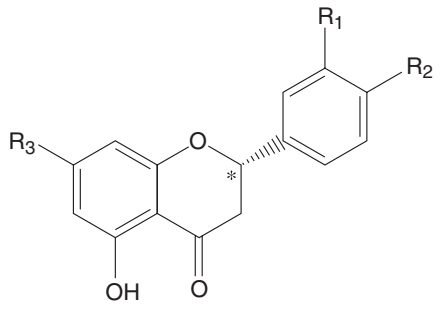


Figure I1.3.5 Structure of flavan-3-ols. Chiral centers indicated by asterisks. G, galloyl.



eriocitrin	$R_1 = R_2 = OH$, $R_3 = O$-rutinoside
eriodictyol	$R_1 = R_2 = R_3 = OH$
hesperetin	$R_1 = R_3 = OH$, $R_2 = OCH_3$
hesperidin	$R_1 = OH$, $R_2 = OCH_3$, $R_3 = O$-rutinoside
naringenin	$R_1 = H$, $R_2 = R_3 = OH$
naringin	$R_1 = H$, $R_2 = OH$, $R_3 = O$-neohesperidoside
narirutin	$R_1 = H$, $R_2 = OH$, $R_3 = O$-rutinoside
neohesperidin	$R_1 = OH$, $R_2 = OCH_3$, $R_3 = O$-neohesperidoside

Figure I1.3.6 Structure of flavanones. Chiral center indicated by an asterisk.

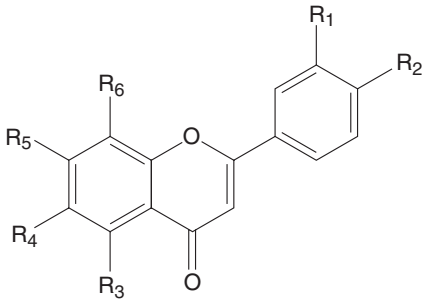
composed of six subgroups: anthocyanidins, catechins (flavan-3-ols), flavonols, flavones, flavanones, and isoflavones. Anthocyanidins and anthocyanins (glycosides or acylglycosides of anthocyanidins) are water-soluble pigments that are mainly responsible for the red, pink, purple, or blue colors in plant materials. They are visible to the human eye and are universal plant colorants. They are usually detected in the visible region of the spectrum between 500 and 530 nm, and are separated in the acidic environment of mobile phases as their flavylium cations. There are six anthocyanidins commonly present in plant-based materials: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Figure I1.3.4B). When using the Basic Protocol, the elution order for anthocyanins and anthocyanidins is kuromanin, malvin, keracyanin, delphinidin, oenin, cyanidin, pelargonidin, peonidin, and malvidin (Table I1.3.1).

Flavanols such as catechin, epicatechin, and epigallocatechin (Figure I1.3.5) are chiefly found in teas (green tea, oolong tea, black tea). Flavanols occur only as aglycone forms, contrary to the fact that most flavonoids exist in plants as glycosides, in which some hydroxyl groups are linked to sugar residues such as glucose, rhamnose, galactose, and arabinose (Lee, 2000). Teas are usually extracted by boiling. Filtration is often the only sample cleanup necessary for HPLC analysis. A typical wavelength for the detection of flavanols is often 210 or 280 nm. When using the Basic Protocol, the elution order for flavanols is epigallocatechin, catechin, epicatechin, epigallocatechin gallate, and epicatechin gallate (Table I1.3.1).

Flavanones (Figure I1.3.6) with a saturated heterocyclic C ring are mainly found in citrus fruits. Natural flavanones have the 2S configuration and usually occur as glycosides, frequently neohesperidosides (2-*O*-α-L-rhamnosyl-D-glucosides) and rutinosides (6-*O*-α-L-rhamnosyl-D-glucosides; Tomás-Barberán and Clifford, 2000). Their glycosylation occurs at the 7 position. Flavanones are generally detected at 280 nm, and possibly at 252, 285, 290, and 365 nm. In reversed-phase liquid chromatography, the elution order of flavanones (by decreasing mobility) is hesperidin, naringin, hesperetin, and naringenin (Swatsitang et al., 2000).

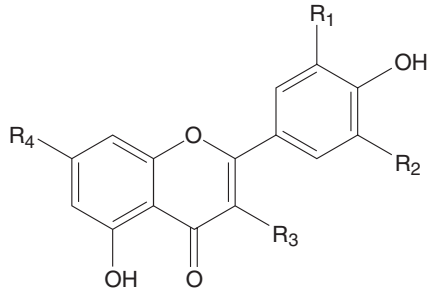
Flavones (Figure I1.3.7) are less commonly found in plants, but often occur in citrus. Polymethoxylated flavones, which include nobiletin, sinensetin, and tangeretin, are the characteristic features in citrus plants. Flavones are generally detected at 360 or 370 nm. The elution order, when analyzed as described in the Basic Protocol, is rhoifolin, luteolin, and apigenin (Table I1.3.1).

Among the flavonoids, flavonols (Figure I1.3.8) are the most prevalent in the plant kingdom. Flavonols have a hydroxyl at the C3 position. They are usually found as glycosides, of which glycosylation occurs at the 3 position



apigenin	$R_1 = R_4 = R_6 = H$, $R_2 = R_3 = R_5 = OH$
luteolin	$R_4 = R_6 = H$, $R_1 = R_2 = R_3 = R_5 = OH$
nobiletin	$R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = OCH_3$
rhoifolin	$R_1 = R_4 = R_6 = H$, $R_2 = R_3 = OH$, $R_5 =$ *O*-neohesperidoside
sinensetin	$R_1 = R_2 = R_3 = R_4 = R_5 = OCH_3$, $R_6 = H$
tangeretin	$R_2 = R_3 = R_4 = R_5 = R_6 = OCH_3$, $R_1 = H$

Figure I1.3.7 Structure of flavones.



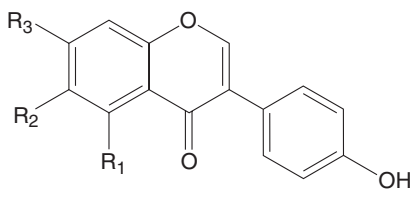
kaempferol	$R_1 = R_2 = H$, $R_3 = R_4 = OH$
myricetin	$R_1 = R_2 = R_3 = R_4 = OH$
quercetin	$R_1 = R_3 = R_4 = OH$, $R_2 = H$
quercitrin	$R_1 = R_4 = OH$, $R_2 = H$, $R_3 =$ *O*-rhamnoside
rutin	$R_1 = R_4 = OH$, $R_2 = H$, $R_3 =$ *O*-rutinoside

Figure I1.3.8 Structure of flavonols.

in plant materials. The three most common aglycones in flavonols are kaempferol, quercetin, and myricetin. Glycosides of quercetin are usually predominant in plants. Flavonols and their glycosides are generally detected at 270, 365, and 370 nm. When analyzed using the Basic Protocol, the elution order for flavonols is rutin, quercitrin, myricetin, quercetin, and kaempferol (Table I1.3.1).

Isoflavones (Figure I1.3.9), which are structural isomers of the previously described flavonoids, are found almost exclusively in soy and soy-based products. The aromatic ring B is linked to the 3 position of the heterocyclic six-membered ring. Isoflavones can be divided into four groups: aglycones, glycosides, 6″-*O*-acetyl-glycosides, and 6″-*O*-malonyl-glycosides. Daidzein, genistein, glycitein, and their glycosides (daidzin, genistin, glycitin) are the major isoflavones. Isoflavones are detected at 260 nm, and possibly between 230 and 280 nm. The elution order for isoflavones by reversed-phase HPLC analysis is daidzin, glycitin, genistin, daidzein, glycitein, and genistein (Griffith and Collison, 2001; Gu and Gu, 2001).

Tannins (Figure I1.3.10) are polyphenolic polymers of high molecular weight that occur naturally and react with proteins. Tannins are



daidzein	$R_1 = R_2 = H$, $R_3 = OH$
daidzin	$R_1 = R_2 = H$, $R_3 = O$-glucoside
genistein	$R_2 = H$, $R_1 = R_3 = OH$
genistin	$R_1 = OH$, $R_2 = H$, $R_3 = O$-glucoside
glycitein	$R_1 = H$, $R_2 = OCH_3$, $R_3 = OH$
glycitin	$R_1 = H$, $R_2 = OCH_3$, $R_3 = O$-glucoside

Figure I1.3.9 Structure of isoflavones.

typically classified into three groups: condensed tannins, hydrolyzable tannins, and phlorotannins. Condensed tannins (often referred to as proanthocyanidins or procyanidins) are oligomers or polymers of flavan-3-ols (e.g., catechins and epicatechins). Hydrolyzable tannins are polymers of gallic or ellagic acid, producing gallotannins or ellagitannins, respectively. Phlorotannins consist of phloroglucinol subunits. HPLC analysis is a useful technique for quantitative or qualitative evaluation of these groups of tannins, although their analysis using HPLC is generally more difficult and complicated than HPLC analyses of phenolic acids and flavonoids. HPLC analysis of these tannins is briefly reviewed elsewhere (Mueller-Harvey, 2001; Schofield et al., 2001).

Because polyphenolics show chemical complexities and similar structures, isolation and quantification of the individual polyphenolic compounds have been challenging. Many traditional techniques (paper chromatography, thin-layer chromatography, column chromatography) have been used. HPLC, with its merits of exacting resolution, ease of use, and short analysis time, has the further advantage that separation and quantification occur simultaneously. A reversed-phase HPLC apparatus equipped with a diode array detector makes possible the easy isolation and separation of many polyphenolics. For enhanced performance of HPLC separation, the polyphenolics should first be isolated into several fractions to effectively separate the individual polyphenolics (Jaworski and Lee, 1987; Oszmianski and Lee, 1990).

Detection of the eluted polyphenolics has been commonly based on the absorptive measurement at characteristic wavelengths. The photodiode array detector has been extensively used for the detection of polyphenolics, mainly because of its collection of online UV-Vis spectra. All polyphenolics absorb in the UV region (Robards and Antolovich, 1997). Examples of UV spectra for polyphenolics are shown in Figure I1.3.11. Two absorption bands are characteristic of flavonoids. Band I, with maximum absorption in the range of 300 to 550 nm, arises from the A aromatic ring (Figure I1.3.4A). Band II, with maximum absorption in the range of 240 to 285 nm, comes from the B ring. Simple phenols, phenolic acids, and hydroxycinnamic acids show their absorption maxima in the range of 230 to 330 nm.

The elution order of polyphenolics may be predicted using a reversed-phase column, which is usually packed with silica-bonded C_8 or C_{18} materials. The more polar polyphenolics are generally eluted first under reversed-phase conditions. Glycosylation in flavonoids increases their polarity and therefore increases their mobility in reversed-phase HPLC. Triglycosides elute before diglycosides, which are followed by monoglycosides and then aglycones. The elution order of benzoic acids, hydroxycinnamic acids, and the aglycones of flavonoids can normally be determined on the basis of the number of polar hydroxyl groups and lipophilic methoxyl groups. The elution order of benzoic acids is as follows: gallic acid, protocatechuic acid, vanillic acid, and syringic acid (Rodríguez-Delgado et al., 2001). The elution order for hydroxycinnamic acids is as follows: caffeic acid, *p*-coumaric acid, sinapic acid, ferulic acid, and cinnamic acid (Schieber et al., 2001). The elution of flavonoids in order

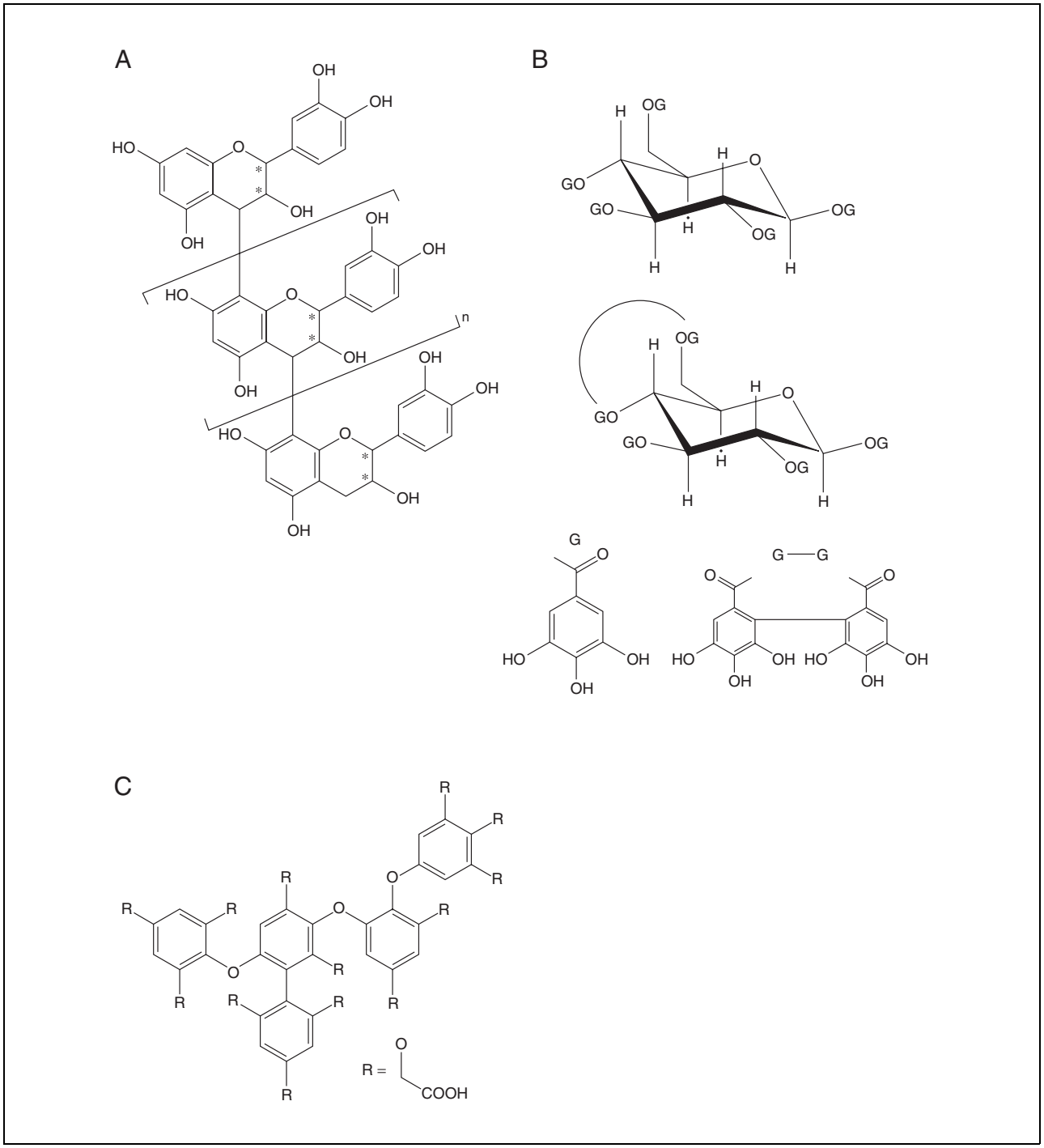


Figure I1.3.10 General structures of (**A**) condensed tannins (n indicates number of subunits), (**B**) hydrolyzable tannins, and (**C**) phlorotannins. Chiral centers indicated by asterisks. G, galloyl.

of decreasing polarity is as follows: catechin, epicatechin, cyanidin, rutin, myricetin, quercetin, and kaempferol. Acylation of polyphenolics reduces their mobility and therefore increases their retention times under the reversed-phase HPLC system.

Mobile-phase elution is frequently a binary system (Merken and Beecher, 2000). Aqueous acetic acid, phosphoric acid, formic acid, perchloric acid, or trifluoroacetic acid is used as an aqueous, acidified polar solvent, whereas methanol or acetonitrile is used as a less-polar solvent. Those acids used as modifiers in an aqueous polar solvent are usually employed for the enhancement of the resolution of peaks. Isocratic elution can be used for polyphenolics that are partially isolated from crude extracts. Gradient elution of mobile phases, which gives

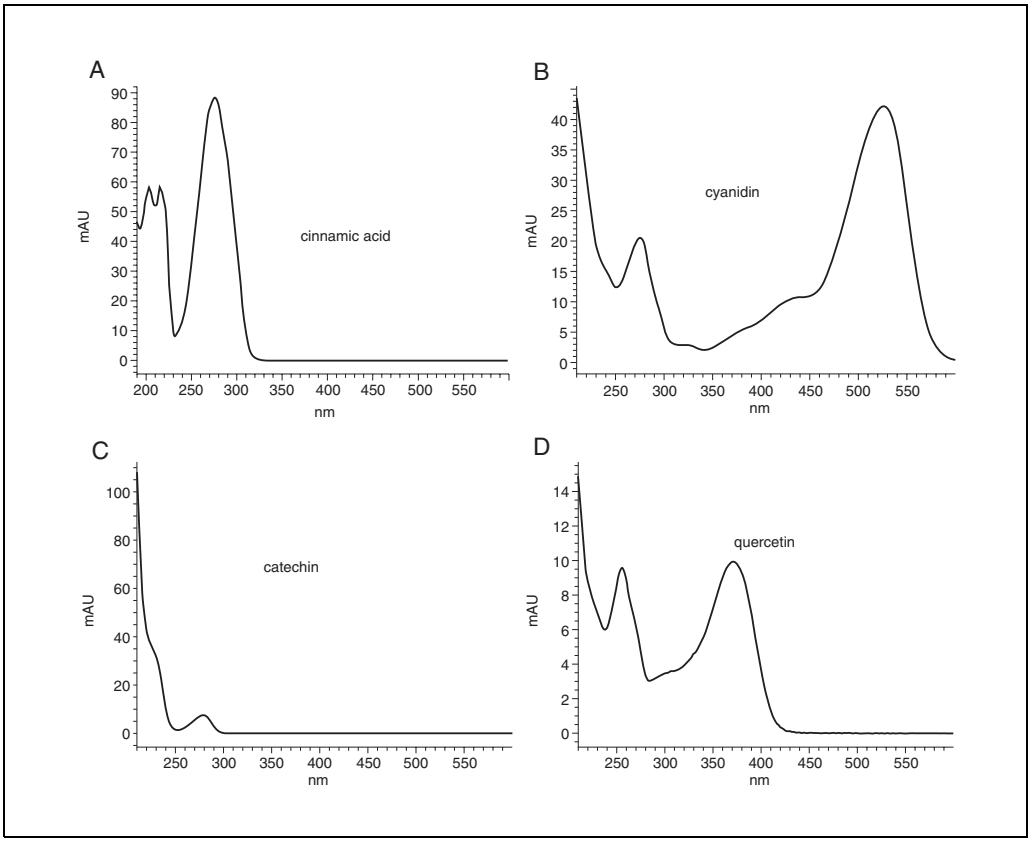


Figure I1.3.11 Sample UV spectra from various polyphenolics classes: (**A**) cinnamic acid (a phenolic acid), (**B**) cyanidin (an anthocyanidin), (**C**) catechin (a flavan-3-ol), and (**D**) quercetin (a flavonol). AU, absorbance units.

enhanced separation efficiency, is employed more commonly in the separation of complex mixtures of polyphenolics from plant materials.

Critical Parameters and Troubleshooting

Reversed-phase HPLC can separate polyphenolics of extracts on the basis of polarity. HPLC easily produces better resolution among chemically similar compounds in extracts than conventional chromatographic methods. The operating temperature of the column during reversed-phase HPLC analysis should be controlled for data reproducibility. A change in temperature produces only a minor effect, however, on band spacing in reversed-phase HPLC and produces essentially no effect in normal-phase HPLC (Lee and Widmer, 1996). A range of ambient temperatures is widely used, and elevated temperatures are often applied. The retention times of the peaks are dependent upon the type of column and the combination of various solvents used in the method.

Samples should be filtered before injection into the HPLC column to prevent it from clogging and thus being damaged. Also, it is recommended that a short guard column be installed before the analytical column. This relatively inexpensive column will capture extract components that may irreversibly adsorb onto the stationary phase of the expensive analytical column. Guard columns need to be replaced before strongly adsorbed contaminants, such as lipids or very hydrophobic molecules, permanently affect the analytical column, causing a loss in column performance. The guard column should be replaced at regular intervals for best results. If column performance deteriorates, the column should be cleaned and regenerated according to the manufacturer's instructions. The reversed-phase column, if not used for a period time, should be saturated and stored with a less-polar solvent such as acetonitrile or aqueous acetonitrile.

Mobile phases containing phosphate are subject to microbial growth, especially molds. These mobile phases should be filter sterilized and stored in autoclaved or sterile containers under refrigeration. Alternatively, a few milligrams of sodium azide per liter of solution may be added to the aqueous mobile phase. An aqueous salt solution used as a mobile phase may cause crystal

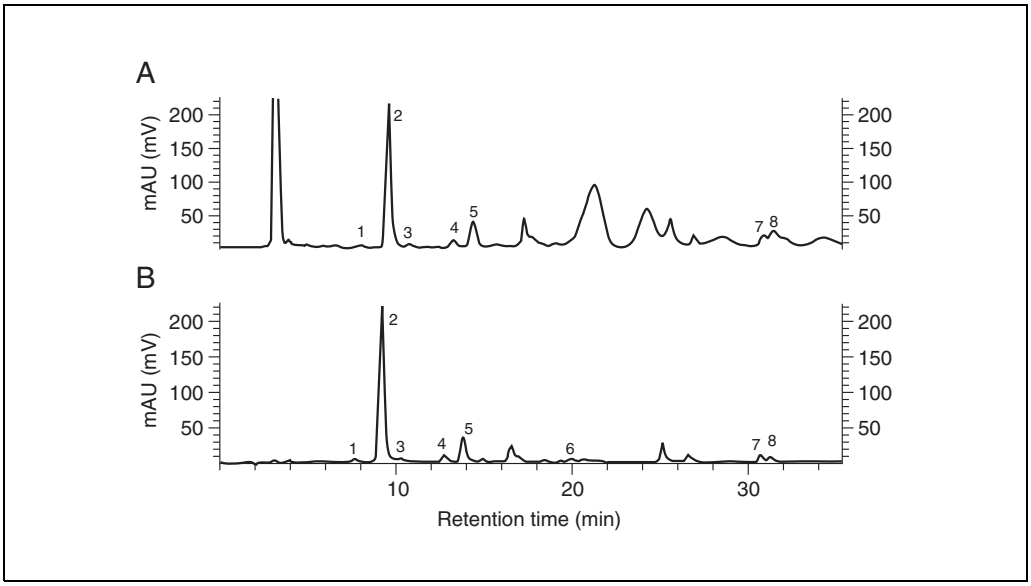


Figure I1.3.12 HPLC chromatograms of polyphenolics in Concord grape extract detected at 280 nm. (**A**) All polyphenolics, including anthocyanins. (**B**) Nonanthocyanin polyphenolics after fractionation. Peak identification: 1, *cis*-caftaric acid; 2, *trans*-caftaric acid; 3, procyanidin B3; 4, *cis*-coutaric acid; 5, *trans*-coutaric acid; 6, epicatechin; 7, quercetin galactoside; 8, quercetin glucoside. Reproduced from Oszmianski and Lee (1990) with permission from the American Society for Enology and Viticulture.

formation or scaling around tubing, which may lead to channeling. The HPLC system should thus be rinsed with water on a regular basis to maintain quality results. The column should always be equilibrated with the initial mobile phase prior to each sample injection.

An accurate sample weight before extraction and the amount of final extract after sample cleanup should be known for an accurate quantification of phenolic compounds extracted from plant materials by HPLC analysis. The characteristic wavelengths for detection of polyphenolics can be selected at the discretion of the experimenter. The solvent gradients described in the Basic and Alternate Protocols can be modified for better resolution.

The determination of polyphenolics may result in interference due to co-elution of phenolic acids and procyanidins. This problem can be eliminated by fractionation of polyphenolics into acidic and neutral polyphenolics prior to sample injection into the HPLC system. Because the fractionation techniques effectively improve the resolution of many polyphenolic peaks in the reversed-phase HPLC system, it is suggested that further characterization and identification of unknown peaks be conducted by additional methods such as mass spectrometry and nuclear magnetic resonance.

Anticipated Results

In reversed-phase HPLC separation of polyphenolics on the basis of polarity, the elution order of polyphenolics may be predicted. The more-polar polyphenolics are generally eluted first under reversed-phase conditions. Glycosylation in flavonoids increases their polarity and therefore their mobility in the reversed-phase system. The elution order of benzoic acids, hydroxycinnamic acids, and aglycones of flavonoids can normally be determined on the basis of the number of polar hydroxyl groups and lipophilic methoxyl groups. For additional information about elution order for various classes of polyphenolics, see Background Information.

After a sample cleanup using a C18 Sep-Pak cartridge, nonanthocyanin polyphenolics from a crude red grape extract were analyzed by reversed-phase HPLC (Oszmianski and Lee, 1990). The removal of 98% to 99% of anthocyanins was achieved. Typical chromatograms of polyphenolics from Concord grapes with and without the isolation of anthocyanins are shown in Figure I1.3.12, which displays the selective fractionation of anthocyanins and nonanthocyanins.

Using the Alternate Protocol, all major polyphenolic compounds are separated with good resolution, which is due to the effective fractionation of acidic and neutral polypheno-

lics (Jaworski and Lee, 1987; Lee and Jaworski, 1987). The acidic polyphenolics, analyzed by isocratic elution, consisted of *trans*-caftaric acid, *cis*-coutaric acid, and *trans*-coutaric acid, whereas the neutral polyphenolics, analyzed by gradient elution, included catechin, catechin-gallate, epicatechin, procyanidin B1, procyanidin B2, and procyanidin B3. Some portions of anthocyanin pigments were eliminated during C18 Sep-Pak fractionation. The elution of the residual pigments in the deproteinated juices occurred during the later part of the run and caused no interference with the reversed-phase HPLC analysis.

Time Considerations

The sample preparation techniques described in the Basic and Alternate Protocols require ~1 to 3 hr prior to sample injection into the HPLC. No additional time for sample cleanup is necessary if the sample is directly injected into the HPLC column without solid-phase extraction.

The Basic Protocol requires 60 min of running time for HPLC analysis after each injection. The period between 55 and 60 min allows for column equilibration prior to the next injection. Analysis of the acidic fraction described in the Alternate Protocol is completed within 30 min including column equilibration time. HPLC analysis of the neutral fraction described in the Alternate Protocol needs much more time (60 min) for sample runs. It is desirable to analyze the sample on the same day as the extraction to prevent possible polyphenolic deterioration.

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Describes solvent gradient conditions for HPLC analysis of wine polyphenolics.

Lee and Jaworski, 1987. See above.

Thoroughly describes sample cleanup of crude extracts and HPLC analysis of neutral and acidic polyphenolics.

Merken and Beecher, 2000. See above.

Extensively reviews the HPLC analysis of flavonoids.

Oszmianski and Lee, 1990. See above.

Thoroughly describes the effective fractionation technique of separating anthocyanins and nonanthocyanins.

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Proanthocyanidins: Extraction, Purification, and Determination of Subunit Composition by HPLC

Proanthocyanidins are polymeric flavonoid compounds composed of flavan-3-ol subunits (*UNIT I1.3*), and are responsible for bitterness and astringency in some foods and beverages. This unit describes methods for extracting and purifying proanthocyanidins, and for determining their subunit composition by HPLC. Based upon HPLC results, the average degree of polymerization and the conversion yield for purified proanthocyanidins can be determined.

This unit is composed of three separate procedures describing proanthocyanidin extraction from plant tissue (see Basic Protocol 1), purification (see Basic Protocol 2), and subsequent analysis by reversed-phase HPLC (see Basic Protocol 3). These protocols have been developed and used for analysis of grape skins, grape berries, and grape seeds. Without modification, wine, apples, and pears have also been analyzed using these procedures.

EXTRACTION OF PROANTHOCYANIDINS

BASIC PROTOCOL 1

This extraction method utilizes an aqueous acetone system to extract proanthocyanidins from whole plant tissue. Acetone is preferred over other extraction solvent systems (most notably methanol) because of its ability to solubilize proanthocyanidin-containing material that is insoluble in methanol.

Materials

Plant material containing proanthocyanidins
66% (v/v) aqueous acetone, HPLC grade
Nitrogen gas

Suitably sized Erlenmeyer flask with stopper or septum
Aluminum foil
Platform shaker
Büchner funnel
Whatman no. 1 filter paper
Suitably sized round-bottom flask
Rotary evaporator with vacuum pump or water aspirator, 40°C

1. Accurately weigh plant material (within ±0.1%) and place into an Erlenmeyer flask. Record the weight of the sample.

   As a general guideline, and based upon grape tissue, 5 to 10 g of plant tissue is placed into a 250-ml Erlenmeyer flask with 100 ml of extraction solvent. To ensure adequate mixing, Erlenmeyer flasks should not be filled to more than 80% of capacity (i.e., 200 ml in a 250-ml Erlenmeyer flask). An approximate yield for this step is 1 to 2 mg of proanthocyanidin extraction per gram of grape berry weight.

2. Add enough 66% (v/v) aqueous acetone to the Erlenmeyer flask to cover the plant tissue and record the volume to within ±0.5%.
3. Equip the Erlenmeyer flask with a stopper or septum and sparge the extraction system well with nitrogen.
4. Cover the flask with aluminum foil and shake 24 hr at room temperature on a platform shaker.

Contributed by James A. Kennedy
Current Protocols in Food Analytical Chemistry (2002) I1.4.1-I1.4.11

5. Remove the flask from the shaker and separate the proanthocyanidin extract from the solid plant material by filtering the mixture through a Büchner funnel equipped with an appropriately sized Whatman no. 1 filter.

   As a general guideline, and based again upon grape tissue, an extraction using 5 to 10 grams of plant tissue can be filtered using a Büchner funnel with a 6-cm-diameter perforated surface and 7-cm-diameter filter paper.

6. Place the filtered proanthocyanidin extract into an appropriately sized round-bottom flask and remove acetone on a rotary evaporator equipped with a water aspirator or vacuum pump, at 40°C.

   Because proanthocyanidins are susceptible to oxidation, the amount of time the flask is left on the rotary evaporator should be limited to the time necessary to remove the acetone. This is most easily determined by observing the rotary evaporator condenser. Because water has a higher surface tension than acetone, condensation of water is observed as fogging of the condenser portion of the rotary evaporator.

7. Store aqueous proanthocyanidin extract at −20°C or cooler.

   The author generally proceeds directly to purification, but has used grape seed extracts stored for up to one week and seen little degradation.

BASIC PROTOCOL 2

PURIFICATION OF PROANTHOCYANIDINS

This purification step is designed to remove impurities from the proanthocyanidin extract. It utilizes liquid-liquid extraction to remove lipophilic material and monomeric flavan-3-ols, and also adsorption chromatography to remove more hydrophilic material such as organic acids, sugars, and residual flavan-3-ol monomers. Following the steps in this protocol, purified and powdered proanthocyanidins are obtained.

Materials

Aqueous crude proanthocyanidin extract (see Basic Protocol 1)
Appropriate solvents for liquid-liquid extraction (e.g., chloroform and ethyl acetate)
Gel filtration medium (Toyopearl HW-40F; Supelco)
Methanol, HPLC grade
Trifluoroacetic acid (TFA), spectrophotometric grade
66% (v/v) aqueous acetone (HPLC grade) containing 0.1% (v/v) TFA
Dry ice/acetone bath

Separatory funnel
Suitably sized round-bottom flasks
Rotary evaporator with vacuum pump or water aspirator, 40°C
Chromatography column
Freeze drier with freeze-drying flask

Perform liquid extraction

1. Depending on the impurities that are present, perform liquid-liquid extraction of the aqueous proanthocyanidin extract to remove impurities with different solubility properties.

   For example, extract the proanthocyanidin mixture with chloroform to remove chlorophyll, carotenoids, and waxy material. Use ethyl acetate if substantial amounts of flavan-3-ol monomers are present. Tissues that would benefit from a chloroform extraction include leafy tissues that contain chlorophyll (i.e., tea leaves) and seeds that contain oils (i.e., grape seeds). Ethyl acetate would be useful in plants such as apples, berries, grapes, and teas (i.e., tissues known to contain significant amounts of flavan-3-ol monomers).

   In general, the volume of extraction solvent should be 20% (by volume) of the aqueous proanthocyanidin extract, and the extraction should be repeated five times.

2. Briefly remove residual organic solvent on a rotary evaporator equipped with a water aspirator or vacuum pump, at 40°C.

Perform adsorption chromatography

3. Prepare a slurry containing a suitable amount of gel filtration medium in 50% (v/v) aqueous methanol containing 0.1% (v/v) TFA, and pack a suitably sized column with this slurry according to the manufacturer's instructions.

   As a rough guideline, approximately 1 gram of proanthocyanidin can be purified from a column bed volume of 100 cm^3 resin.

4. Precondition the column with 50% (v/v) aqueous methanol containing 0.1% (v/v) TFA.

5. Add methanol first and then TFA to the aqueous proanthocyanidin extract to obtain a 50% (v/v) methanol concentration with a 0.1% (v/v) TFA concentration. Apply the extract to the column.

6. Rinse the column with at least 5 column volumes of 50% methanol/0.1% TFA solution.

   The potential impurities will vary according to the plant tissue extracted, and therefore the exact washing volume will vary. It is important to determine the impurities present and their retention properties on the column to minimize impurities in the final proanthocyanidin and maximize proanthocyanidin recovery. For this step, the use of a spectrophotometer is helpful in monitoring the eluate. Some typical impurities and monitoring wavelengths include organic acids (215 nm), flavan-3-ol monomers (280 nm), hydroxycinnamic acids (320 nm), and flavonols (365 nm). Anthocyanins are observable in the visible spectrum.

7. Elute the adsorbed proanthocyanidins with 2 column volumes of 66% (v/v) acetone/0.1% TFA.

   This volume is normally sufficient to elute the adsorbed proanthocyanidins.

8. Transfer the proanthocyanidin-containing eluate into an appropriately sized and preweighed round-bottom flask and remove the acetone on a rotary evaporator.

9. Freeze the aqueous proanthocyanidin-containing solution in a dry ice/acetone bath.

   When freezing, and to minimize the time necessary to freeze dry the sample (next step), maximize the surface area of contact between the flask and the dry ice. This is most easily achieved by rotating the flask within the dry ice/acetone mixture so that the walls of the round-bottom flask are coated with the ice.

10. Transfer to a freeze-drying flask and freeze dry the aqueous proanthocyanidins.

11. Weigh the proanthocyanidins and calculate recovery in mg proanthocyanidins/kg sample (see Basic Protocol 1, step 1).

12. Store proanthocyanidins in a light-protected container at −20°C or cooler.

    Proanthocyanidins will slowly oxidize under freezer conditions. Based upon the storage of grape seed proanthocyanidins at −20°C, ~95% of the initial proanthocyanidins remain after 6 months of storage.

DETERMINATION OF SUBUNIT COMPOSITION BY HPLC

In this protocol, the subunit composition and the conversion yield of purified proanthocyanidins is determined by reversed-phase HPLC. The instrument has been selected because of its ability to run a gradient and because it can acquire UV/Vis spectra, which can be useful for compound identification.

In the initial stages of this analytical method the proanthocyanidins are cleaved by acid catalysis into their constitutive subunits. Proanthocyanidin extension subunits, upon cleavage, form unstable electrophilic intermediates. Phloroglucinol (1,3,5-trihydroxybenzene) is added to the reaction mixture as a nucleophile, where it combines with extension subunit intermediates to form analyzable adducts.

This method analyzes proanthocyanidins after cleavage, as opposed to analyzing intact proanthocyanidins, for the following reasons: cleavage reactions provide compositional information and unambiguous identification of proanthocyanidin-derived material as well as providing information on the proportion of purified material that is unknown. Due to the inherent reactivity and heterogeneity of proanthocyanidins, chromatographic methods that analyze the material while still intact provide only approximate quantitative information and are nebulous in terms of providing compositional information.

Materials

Purified proanthocyanidins (see Basic Protocol 2)
Phloroglucinol solution (see recipe)
40 mM sodium acetate buffer (see recipe)
Mobile phase A: 1% (v/v) acetic acid in HPLC-grade water
Mobile phase B: HPLC-grade methanol
(+)-Catechin hydrate (Sigma)

2-ml borosilicate glass vials with a caps
Water bath at 50°C
High performance liquid chromatograph (HPLC) with:
HPLC vials
250 × 4.6–mm Wakosil II 5C18 column (5-µm particle size) and guard column (SGE)
UV absorption detector set at 280 nm

Table I1.4.1 Retention Properties, Molar Absorptivities, and Response Factors for Common Proanthocyanidin Cleavage Products[a]

Compound	Retention factor (k)	Molar absorptivity (ε_{280})[b]	Relative molar response[c]	Corrected relative mass response[c,d]
(–)-Epigallocatechin-(4β→2)-phloroglucinol[e]	5.8	1344	0.34	0.32
(–)-Epicatechin-(4β→2)-phloroglucinol[e]	9.4	4218	1.06	1.06
(+)-Catechin-(4α→2)-phloroglucinol[e]	9.1	4218	1.06	1.06
(–)-Epicatechin-3-*O*-gallate-(4β→2)-phloroglucinol[e]	13.4	14766	3.70	2.44
(–)-Epicatechin[f]	16.6	3988	1.00	1.00
(+)-Catechin[f]	13.1	3988	1.00	1.00
(–)-Epicatechin-3-*O*-gallate[f]	19.1	12611	3.16	2.07

[a]Reprinted from Kennedy and Jones (2001) with permission from the American Chemical Society.
[b]In methanol.
[c]Relative to catechin.
[d]Not including the phloroglucinol moiety.
[e]Product derived from proanthocyanidin extension subunit.
[f]Product derived from proanthocyanidin terminal subunit.

1. Place 5 mg purified proanthocyanidins into a clean 2-ml borosilicate glass vial.
2. Add 1 ml phloroglucinol solution, cap the vial, and dissolve the proanthocyanidins.
3. Place the proanthocyanidin solution into a water bath preheated to 50°C and allow acid catalysis to proceed for 20 min.
4. Remove the vial from the water bath and allow the contents to return to room temperature.
5. Transfer 1 ml of 40 mM sodium acetate buffer to an HPLC vial and add 200 µl proanthocyanidin solution .
6. Inject 20 µl onto an HPLC equipped with a 250 × 4.6–mm, 5-µm Wakosill 5C18 column and a guard column containing the same material. Separate proanthocyanidin subunits according to manufacturer's instructions, with peak detection at 280 nm and the following linear gradients at a flow rate of 1.0 ml/min:

   5% (v/v) mobile phase B for 10 min
   5% to 20% mobile phase B in 20 min
   20% to 40% mobile phase B in 25 min.
7. Before the next injection, wash the column with 90% mobile phase B for 10 min and reequilibrate with 5% mobile phase B for 5 min.
8. Identify proanthocyanidin subunits by comparing their retention factors to those shown in Table I1.4.1. After identification, quantify using a (+)-catechin standard.

   Subunits that react with phloroglucinol are derived from proanthocyanidin extension subunits. Subunits that have not reacted with phloroglucinol are derived from proanthocyanidin terminal subunits or were present as monomeric flavan-3-ols. For quantitation, the sample peak areas of the individual subunits are compared with a (+)-catechin standard, and individual quantities are determined using the relative response factors shown in Table I1.4.1.
9. Determine the conversion yield by summing the mass of the subunits (not including the phloroglucinol moiety) and dividing by the starting mass of proanthocyanidins:

$$\text{conversion yield} = \frac{\left(\frac{406\ \text{PAU}}{0.32} + \frac{68\ \text{PAU}}{1.06} + \frac{1816\ \text{PAU}}{1.06} + \frac{264\ \text{PAU}}{1.00} + \frac{154\ \text{PAU}}{2.44}\right) \times \left(\frac{96\ \text{mg/l}}{460\ \text{PAU}}\right) \times \left(\frac{6}{1000}\right)}{5.04\ \text{mg sample}} = 0.85$$

   In this example, individual subunit peak areas (PAU) are divided by their relative mass response (Table I1.4.1); 96 mg/liter and 460 PAU are the concentration and peak area of (+)-catechin, and 6/1000 is the dilution factor.
10. Determine the average degree of polymerization by summing the subunits (in mole equivalents) and dividing by the sum of the terminal subunits (also in mole equivalents):

$$\text{average degree of polymerization} = \frac{\left(\frac{406\ \text{PAU}}{0.34} + \frac{68\ \text{PAU}}{1.06} + \frac{1816\ \text{PAU}}{1.06} + \frac{264\ \text{PAU}}{1.00} + \frac{154\ \text{PAU}}{3.70}\right)}{\frac{264\ \text{PAU}}{1.00}} = 12.41$$

   In this example there is only one terminal subunit.

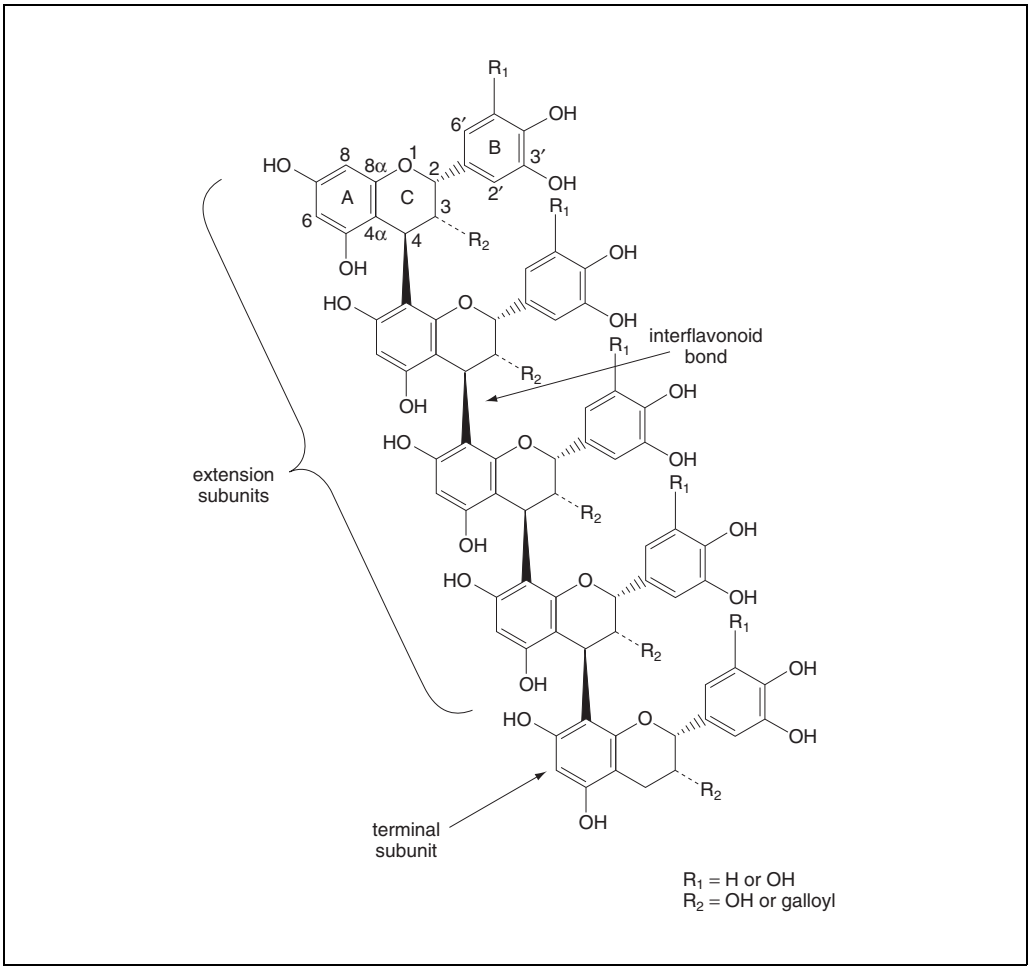


Figure I1.4.1 Generalized proanthocyanidin structure indicating subunit type (extension or terminal) and interflavonoid bond location (4β→8). The most common proanthocyanidin classes in the plant kingdom, as well as in the food and beverage industry, are the procyanidins (3,3′,4′,5,7-pentahydroxyflavans) and prodelphinidins (3,3′,4′,5′,5,7-hexahydroxyflavans). In addition, these proanthocyanidins can be galloylated at C3.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Phloroglucinol solution

Dissolve 5 g phloroglucinol (1,3,5-trihydroxybenzene) and 1 g ascorbic acid in a minimum amount of methanol (60 ml) and transfer to a 100-ml volumetric flask. Add 0.5 ml of 10 N hydrochloric acid, fill to the mark with methanol, and mix well. Store up to 2 weeks at 4°C. Bring to room temperature before use.

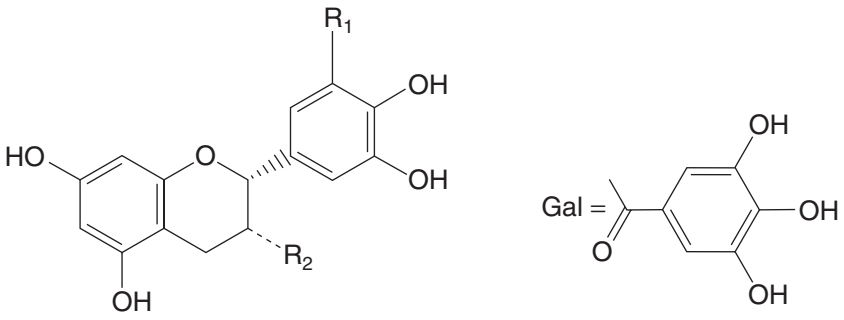
Up to 100 samples can be analyzed with this volume of solution.

CAUTION: *Hydrochloric acid is very corrosive, and 10 N hydrochloric acid releases hydrochloric acid gas when exposed to air. Wear goggles, gloves, and protective clothing, and measure in a well-ventilated area, preferably a fume hood.*

Sodium acetate buffer, 40 mM

Dissolve 0.33 g sodium acetate into a 100-ml volumetric flask filled with approximately 50 ml water. After dissolution, fill to the mark with water and mix well. This solution is intended for immediate use and should be used within 1 day.

Up to 100 samples can be analyzed with this volume of solution.



R_1	R_2	Subunit
H	OH	(+)-catechin
H	OH	(–)-epicatechin
H	OGal	(–)-epicatechin-3-*O*-gallate
OH	OH	(+)-gallocatechin
OH	OH	(–)-epigallocatechin
OH	OGal	(–)-epigallocatechin-3-*O*-gallate

Figure I1.4.2 Common flavan-3-ol subunits found in proanthocyanidins in foods and beverages.

COMMENTARY

Background Information

Proanthocyanidins are polymeric flavonoid compounds composed of flavan-3-ol subunits (Fig. I1.4.1), and are widely distributed in the plant kingdom, including plants that are important as a source of food (Santos-Buelga and Scalbert, 2000). They impart bitter and astringent properties. In addition, these compounds may have potential health effects (Santos-Buelga and Scalbert, 2000).

Proanthocyanidins are so named because under oxidative and acidic conditions they are converted to anthocyanidins, a subclass of flavonoid (for general structure, see Fig. I1.3.4). Historically, they have also been referred to as leucoanthocyanidins, condensed tannins, or simply tannins because of their ability to fix or "tan" leather hides.

Several flavan-3-ol subunits are found in food-based proanthocyanidins, with the most common flavan-3-ol subunit being (–)-epicatechin (Fig. I1.4.2). Flavan-3-ol subunits are linked together by various interflavonoid bonds, with the 4β→8 interflavonoid bond being the most common, followed by the 4β→6 interflavonoid bond. Other types of interflavonoid bonds are also present, although much less common (Hemingway, 1989a). Proanthocyanidins encompass a very large range of molecular weights ranging from the simplest dimer to proanthocyanidins that reportedly exceed 80 subunits in length.

To analyze proanthocyanidins, this unit relies on the susceptibility of the proanthocyanidin interflavonoid bond to acid catalysis. Interflavonoid bonds have different susceptibilities to acid catalysis (Hemingway and McGraw, 1983; Beart et al., 1985). In general, and for procyanidins and prodelphinidins, subunits that are bonded through the benzylic position (C4) are susceptible to acid catalysis. Most natural proanthocyanidins extracted from plant tissue have this type of interflavonoid bond and are therefore suitable candidates for this analytical technique.

Traditional methods for analyzing proanthocyanidins have relied on general colorimetric methods. The main drawback for using these analytical methods is that they are not specific for proanthocyanidins. This can be said for chromatographic methods that analyze intact proanthocyanidins. The selectivity of the

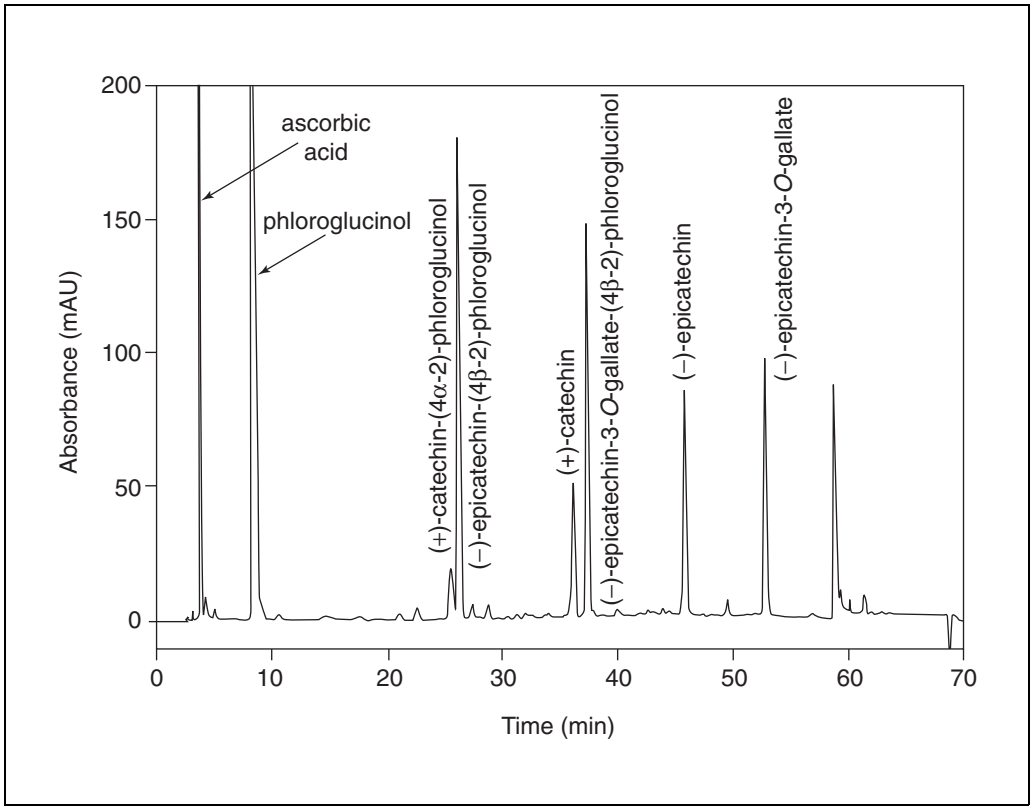


Figure I1.4.3 Sample chromatogram of proanthocyanidin cleavage products from grape seed. Reprinted from Kennedy and Jones (2001) with permission from the American Chemical Society.

method presented here relies on two features of proanthocyanidins: susceptibility to acid-catalyzed cleavage and subsequent attack by strong nucleophiles. Therefore, the products that are formed from acid catalysis in the presence of a nucleophile (phloroglucinol) are clearly derived from proanthocyanidins, and this is the primary advantage that this method has over other analytical methods that are used to measure proanthocyanidins.

Historically, two nucleophiles have been used to trap the cleavage intermediates: benzyl mercaptan and phloroglucinol. Benzyl mercaptan has the distinct disadvantage of having a powerful stench, and therefore it is necessary to conduct reactions involving this reagent in a fume hood. The advantage of using benzyl mercaptan was thought to be the higher conversion yield of proanthocyanidins into their constitutive subunits (Matthews et al., 1997); however, if proper conditions are selected, phloroglucinol is as effective (Kennedy and Jones, 2001).

Critical Parameters and Troubleshooting

Extraction

The extraction of proanthocyanidins is the first step in determining their subunit composition. A number of extraction systems have been investigated in different plant tissues. The most common solvent systems are acetone and methanol with various amounts of water and with or without acid. In general, it has been found that an aqueous acetone system gives the best results in terms of total amount extracted.

Because of the general reactivity of proanthocyanidins (Hemingway, 1989b; McGraw, 1989; Laks, 1989), it is expected that they will quickly become modified once they are extracted into foodstuffs. Specifically, proanthocyanidins are very susceptible to oxidative degradation, and therefore consideration should be given to minimizing oxidation reactions from extraction to analysis. Reduced temperatures and dark conditions are desirable for minimizing oxidation reactions (Cork and Krockenberger, 1991).

In many foods, the oxidation of proanthocyanidins is desirable. Two notable examples are black teas and cocoa. The oxidative modifica-

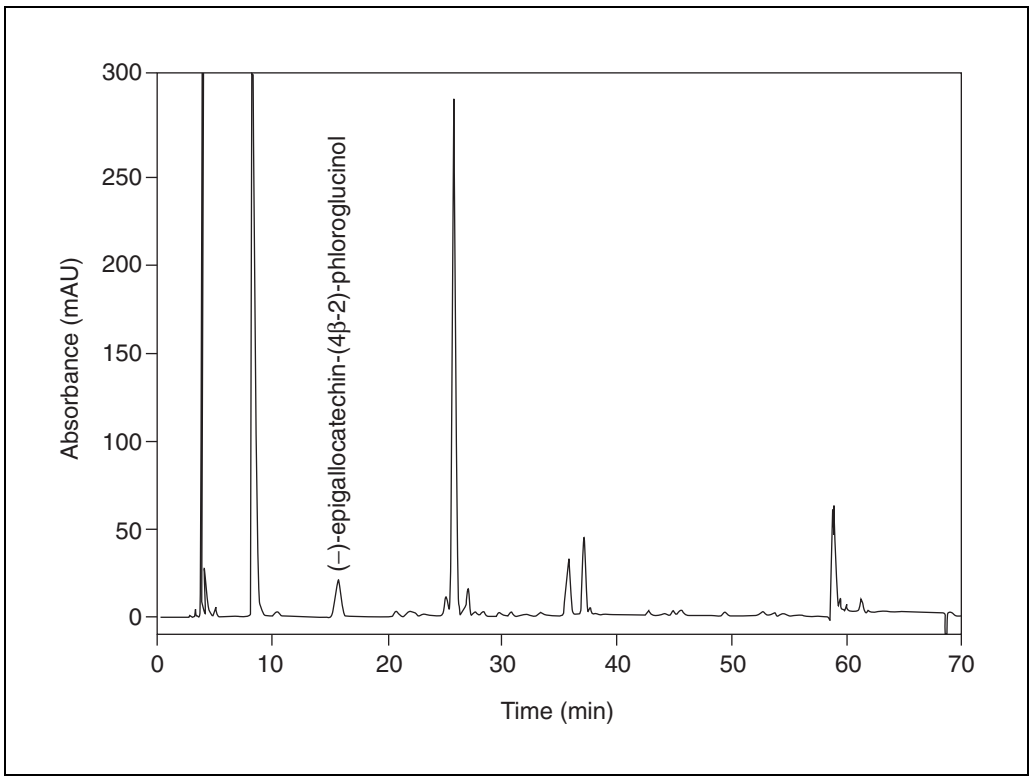


Figure I1.4.4 Sample chromatogram of proanthocyanidin cleavage products from grape skin. Peak identification is identical to Figure I1.4.3, with (–)-epigallocatechin-(4β→2)-phloroglucinol also identified. Reprinted from Kennedy and Jones (2001) with permission from the American Chemical Society.

tion of proanthocyanidins in these foods is considered beneficial because it reduces proanthocyanidin bitterness and astringency. The oxidative products formed from this reaction are resistant to acid catalysis and, as a result, the conversion yield declines with oxidation. For this reason, this analytical method is ideally suited for the analysis of proanthocyanidins in plant tissues, and also in foodstuffs, particularly when oxidation is being monitored.

It is important to determine whether the plant tissues should be extracted whole and for how long. Homogenizing the plant tissue in the extraction solvent will significantly reduce the time needed to complete the extraction; however, this could also lead to a reduction in recovery due to the introduction of impurities and potential loss of proanthocyanidins from complexation and subsequent precipitation with polysaccharides and proteins. In grape seeds, homogenization reduces recovery. This has been attributed to protein precipitation by seed proteins. Cryogenic milling (*UNIT F1.1*) of the plant tissue prior to extraction is an alternative to homogenization but has the same potential pitfalls. Leaving the plant tissue intact can minimize the introduction of impurities and complexation reactions, but then the extraction time must be increased. The selection of optimal conditions is tissue specific, and should be determined in initial runs.

Purification and subunit composition

It is important to purify proanthocyanidins, particularly for determining their conversion yield. It is also advantageous to do so to eliminate extraneous material that might otherwise react with the proanthocyanidins. A combination of liquid-liquid extraction and adsorption chromatography is effective in removing impurities. The use of chloroform in liquid-liquid extraction is very effective in removing fat-soluble compounds such as carotenoids, chlorophyll, oils, and waxes. These compounds would be expected in leafy plant tissues (carotenoids and chlorophyll) as well as seeds and fruits (oils and waxes). Ethyl acetate is effective in the selective removal of flavan-3-ol monomers, which are also typically present with proanthocyanidins.

It is critically important that the purified proanthocyanidins do not contain water prior to acid-catalyzed cleavage with phloroglucinol. The cleavage of proanthocyanidins into

their constitutive subunits generates electrophilic extension subunit intermediates. In this reaction, phloroglucinol, because of its nucleophilicity, is used as a trap to stabilize the extension subunits. Water, if present, competes with phloroglucinol in this reaction, and thus reduces conversion yields, and can also effect the calculated composition of the proanthocyanidin isolate.

When analyzing the cleavage products, it is possible to obtain false positives, specifically with the terminal subunits that will be present as flavan-3-ol monomers. If purification is incomplete, free flavan-3-ol monomer impurities will be present. Although these components are often of interest analytically, they are not, strictly speaking, proanthocyanidins. Analyzing the purified proanthocyanidin before and after acid catalysis in the presence of excess phloroglucinol using the same HPLC method will give some indication of whether or not flavan-3-ol monomers are present.

It is important to monitor the time that the proanthocyanidin is allowed to react with the phloroglucinol solution. The products formed are not stable under acidic conditions, and it is therefore critically important that the reaction not exceed 20 min. Of particular concern are the flavan-3-ol monomers, which degrade more rapidly than the phloroglucinol adducts (Kennedy and Jones, 2001). Excessive degradation of the flavan-3-ol monomers will result in reduced amount of terminal subunits. This in turn will reduce the conversion yield and increase the average degree of polymerization calculated.

Anticipated Results

Extraction

Because of the varied nature of the plant tissues from which the proanthocyanidin extracts are derived, it is difficult to anticipate the expected outcome. As an example of how these procedures can be adapted to specific tissues and analyses, using grape tissues, fruit is harvested and the tissues of interest (e.g., skins and seeds) are removed from the remainder of the berry. They are rinsed well and then extracted as whole tissues using the conditions described in these protocols. For grape skins, a liquid-liquid extraction with chloroform has been successful in the removal of chlorophyll and waxes, yet no extraction with ethyl acetate has been performed because of the small proportion of flavan-3-ol monomers (Kennedy et al., 2001). For grape seeds, these protocols have been adapted to minimize purification and have been used to monitor proanthocyanidin development (Kennedy et al., 2000).

Purification

Proanthocyanidins following purification are light buff powders, with a varying degree of yellow depending on the level of oxidation that has occurred.

Proanthocyanidins are very susceptible to oxidative degradation. To minimize subsequent oxidation, proanthocyanidins should be stored protected from light and under freezer conditions (–20°C or cooler).

Subunit composition

Example chromatograms are shown in Figures I1.4.3 and I1.4.4. Generally speaking, epicatechin extension subunits are the most prevalent subunits found in plant tissues of interest as foodstuff. Some additional references are given at the end of this unit that provide information on the subunit composition of various plant species.

The expected conversion yield can vary considerably. Proanthocyanidins that are isolated from immature plant tissues during active growth and are judiciously protected from oxidation should have high conversion yields, approaching quantitative conversion. Proanthocyanidins isolated from fully ripened plant tissue or fully senesced plant tissues will have lower conversion yields (roughly 50% to 80% by mass). Conversion yields will also be lower if oxidation has occurred as a result of food processing or prolonged storage.

Time Considerations

Extraction

Extraction time will vary, but as this procedure is written for extraction of whole plant tissue, allow for ~26 hr between the time the extraction is set up and the removal of the acetone following extraction. The extraction time for homogenized or milled tissues is typically less than 15 min per sample.

Purification

Allow 12 to 18 hr to purify proanthocyanidins on the column. Drying time will vary depending on the volume obtained and the freeze-drier that is used.

Subunit composition

The analyst should allow ~5 min per sample for weighing and diluting samples with sodium

acetate buffer, followed by 20 min for the catalysis reaction to occur. In addition, each sample run requires 70 min on the HPLC. An experienced analyst should be able to analyze 20 samples and standards in a day.

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Kennedy and Jones, 2001. See above.

Analytical considerations for this unit are detailed in this reference.

Santos-Buelga and Scalbert, 2000. See above.

This review article discusses the presence of proanthocyanidins in foodstuffs and their potential effects on human health.

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Oregon State University
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Identification of Flavonol Glycosides Using MALDI-MS

Flavonol glycosides are found ubiquitously throughout higher plants. They are responsible for the yellow pigmentation of many fruits, vegetables, and grains. The flavonol glycoside composition of plants is distinctive, and thus can provide valuable information for taxonomic purposes and authentication of processed food products.

Measurement of specific flavonol glycosides in complex mixtures can sometimes be difficult. A new analytical technique that shows promise in this area is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS). This technique has proven successful for identifying flavonol glycosides in a number of food sources including green tea, onions, and almonds (Wang and Sporns, 2000; Frison-Norrie and Sporns, 2002). One major advantage of MALDI-MS is that only rudimentary purification of the sample is required prior to analysis. Also, the analysis is very rapid, generally 1 to 2 min per sample run. Fragmentation is minimal, allowing the molecular weight of the parent ions to be easily determined. Characteristic loss of carbohydrate residues in succession from flavonol glycosides can provide structural insight. Flavonol glycosides are easily ionized and have been shown to out-compete impurities for ionization energy, resulting in the appearance of only matrix peaks and flavonol glycoside peaks in the mass spectrum. However, MALDI-MS is unable to differentiate among structural isomers with identical molecular weights, and can be subject to a high degree of variability among identical runs.

This unit describes procedures for extraction, purification, and identification by MALDI-MS of flavonol glycosides from a plant source. The extraction and purification protocols are not meant to be comprehensive, but rather to offer guidelines for sample preparation prior to a MALDI-MS analysis. The MALDI-MS technique is suggested as a complement to other analytical methods such as HPLC or NMR. Its strength lies in the ability to rapidly screen a number of samples for the presence of flavonol glycosides, which can be identified on the basis of their molecular weights.

EXTRACTION OF FLAVONOL GLYCOSIDES FROM PLANT SOURCES

BASIC PROTOCOL 1

Methanol is a common solvent for extracting flavonol glycosides from plant material. This procedure involves a simple, rapid, and efficient extraction whereby powdered plant material is stirred with 70% methanol, filtered, and concentrated on a rotary evaporator. As this extraction is not selective, the crude aqueous extract contains contaminants co-extracted with flavonol glycosides. In most cases, however, the impurities do not detract from the quality of the MALDI-MS results.

Materials

Powdered plant material (freeze-dried if fresh sample has high water content, e.g., >30%)
70% (v/v) methanol in water
Whatman no.1 filter paper
Tapered glass funnel
250-ml round-bottom flask
Rotary evaporator with vacuum pump or water aspirator, 35°C

1. Mix ~5 g powdered plant material (accurately weighed and recorded) with 100 ml of 70% methanol using a chemical-resistant stir bar. Stir 30 min.

Contributed by Suzanne Frison and Peter Sporns
Current Protocols in Food Analytical Chemistry (2002) I1.5.1-I1.5.8

Half an hour should be sufficient time for extraction of flavonol glycosides because of the high surface area of powdered material and the large volume of solvent. Another method of extraction is to add solvent to the sample in two to three aliquots, decant each through Whatman no. 1 filter paper at the end of a specified time, and combine the filtrates.

Flavonol glycosides are minor constituents in foods, likely constituting less than 1% of the dry weight. In order to achieve sufficient concentration in the extract to detect flavonol glycosides in high-moisture foods (30%) without requiring large volumes of samples and extraction solvents, high-moisture samples such as fruit and leaf material should be freeze-dried first.

2. Separate the flavonol glycoside extract from the insoluble plant material by filtering the slurry by gravity through a Whatman no. 1 filter paper into a 250-ml round-bottom flask.

3. Remove methanol in a rotary evaporator at 35°C under vacuum.

   The presence of flavonol glycosides is indicated by the yellow color of the solution.

   A noticeable reduction in the rate of evaporation and an apparent increase in viscosity indicate that the residual liquid is mainly water. Evaporating to dryness should be avoided, or material may become tightly stuck to the flask.

4. Bring the remaining aqueous extract to a known volume (25 ml) with deionized distilled water. Store for up to 1 to 2 weeks at 4°C.

BASIC PROTOCOL 2

PURIFICATION OF FLAVONOL GLYCOSIDES FOR MALDI-MS ANALYSIS

Although MALDI-MS can be quite tolerant of impurities, a purification step is often necessary after extraction in order to enhance the response. Considerable amounts of co-extracted material may compete for ionization energy and mitigate the flavonol glycoside signal. Solid-phase extraction using a C18 cartridge is used to enrich the concentration of flavonol glycosides by removing sugars, acids, and other water-soluble impurities. The final methanolic elution may remove other hydrophobic or phenolic compounds along with flavonol glycosides from the column, but the sample will in most cases be sufficiently clean to obtain a significant flavonol glycoside response with MALDI-MS.

Materials

Aqueous flavonol glycoside extract (see Basic Protocol 1)
Methanol
70% (v/v) methanol in water (or 0.01 M NaCl in 70% methanol)
0.22-µm syringe filters
C18 cartridge (e.g., C18 Sep-Pak cartridge, 360 mg sorbent, Waters Chromatography)

1. Filter sample through a 0.22-µm membrane.

2. Condition a C18 cartridge by passing five column volumes of methanol through sorbent bed.

3. Pass five column volumes of deionized distilled water through cartridge to remove remaining methanol.

4. Load 5 ml aqueous flavonol glycoside extract onto the column. Load at a rate of ~1 ml/min to ensure complete adsorption of flavonol glycosides.

   The volume of extract applied to the cartridge depends on the sample concentration, flavonol glycoside concentration, and amount of sorbent packing in the column.

The column packing will become colored. If excessive color leaks through the cartridge, the cartridge has become overloaded and no more sample should be applied.

5. Wash cartridge with 30 column volumes deionized distilled water to remove water-soluble impurities (sugars, acids).

6. Elute flavonol glycosides with 2 ml of 70% methanol. Add solvent at a rate of ~1 ml/min to ensure complete elution of flavonol glycosides from the cartridge.

   Sodium chloride may be added to the eluting solvent at a concentration of 0.01 M in order to accentuate sodium adduct ions in the MALDI-TOF mass spectrum and suppress the formation of potassium adduct ions.

7. Store purified flavonol glycoside extract up to 1 to 2 weeks at 4°C.

MALDI-MS IDENTIFICATION OF FLAVONOL GLYCOSIDES

BASIC PROTOCOL 3

A high-intensity pulse of UV light initiates the matrix-mediated ionization and vaporization of analyte molecules. Analytes undergo only minimal fragmentation by this soft ionization process, resulting in the appearance of abundant molecular ions in the mass spectrum. Only singly charged positive ions are formed, allowing rapid identification of flavonol glycosides based on molecular weight.

Materials

Matrix solution: 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP; Aldrich) dissolved in acetone at 2 mg/100 µl, freshly prepared
Purified aqueous flavonol glycoside extract (see Basic Protocol 2)
0.2 to 2 µl digital micropipettor or microsyringe
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) with UV laser (i.e., 337-nm nitrogen laser), MALDI probe, and delayed extraction or reflectron

NOTE: In solution, THAP degrades rapidly at room temperature (within 1 to 2 days). Matrix solution should be made fresh daily.

1. Using a microsyringe or digital micropipettor, load 0.5 µl matrix solution onto each position on the MALDI probe. Allow solvent to evaporate (requires only a few seconds).

   Loading more than 1 µl may cause solution to run outside of the boundaries on the probe.

2. Load 0.5 µl purified aqueous flavonol glycoside extract on top of matrix and allow to air dry (requires ~2 min with a fan).

   Many spectra can be obtained from a single position. However, sometimes non-uniformity in crystallization can prevent a satisfactory spectrum from being generated. For this reason, at least two positions per sample should be spotted to allow a better chance of obtaining an acceptable spectrum.

   To encourage homogeneous crystallization, the sample should be dissolved in an aqueous solution (30% aqueous or more). Samples prepared in high organic solvent concentrations may redissolve the THAP crystal bed, which causes irregular recrystallization. Non-uniform crystal formation is one cause of variability among identical runs.

   Alternatively, the sample and matrix solutions can be mixed 1:1 (v/v) and aliquots of up to 1 µl spotted onto the probe.

3. Insert probe into ion source of the MALDI-MS instrument.

4. Record MALDI mass spectra in positive ion mode. Look for proton, sodium, and potassium adduct ions in the range *m/z* 250 to 1000. Fragment ions caused by the loss of carbohydrate moieties are typical and can be diagnostic.

For good resolution and signal-to-noise ratios, laser strength should be slightly above the threshold required to generate detectable ions.

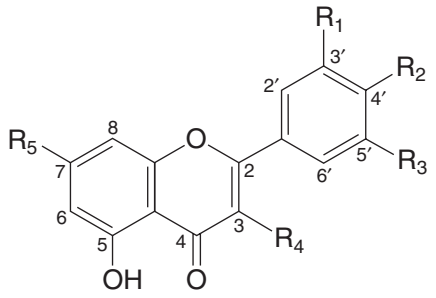
Fragmentation patterns of standards can help to determine whether observed peaks are the result of in-source fragmentation or natural occurrence in the sample. Flavonol glycoside standards are available from Extrasynthese and Indo Fine Chemical.

If flavonol glycosides were eluted with excess sodium, potassium adduct ions will likely be suppressed, but there may be the formation of $[M+2Na-H]^+$ ions.

COMMENTARY

Background Information

Flavonol glycosides are water-soluble pigments responsible for the yellow hues of many fruits, vegetables, and cereal grains. It is also thought that consumption of these phytochemicals may confer protective effects against chronic diseases due to their antioxidant properties (Pietta, 2000). Analyzing flavonol glycosides is useful for taxonomic purposes or for authentication of processed products, because the flavonol glycoside composition of individual plants is distinctive. There is considerable structural variability among flavonol glycosides (Fig. I1.5.1). The most common flavonol aglycones are quercetin, kaempferol, and myricetin, with minor variations on these basic structures (i.e., variations in methylation and hydroxylation patterns) encountered more rarely. Sugar residues may occur at any of the hydroxylated positions, although glycosylation



Compound	Mass	R_1	R_2	R_3	R_4	R_5
Astragalin	448.1	H	OH	H	OGlu	OH
Hyperoside	464.1	OH	OH	H	OGal	OH
Isoquercitrin	464.1	OH	OH	H	OGlu	OH
Isorhamnetin	316.1	OMe	OH	H	OH	OH
Kaempferide	300.1	H	OMe	H	OH	OH
Kaempferol	286.1	H	OH	H	OH	OH
Myricetin	318.0	OH	OH	OH	OH	OH
Narcisin	624.2	OMe	OH	H	ORut	OH
Peltatoside	596.1	OH	OH	H	OAraGlu	OH
Quercetin	302.2	OH	OH	H	OH	OH
Quercitrin	448.1	OH	OH	H	ORha	OH
Rhamnetin	316.1	OH	OH	H	OH	OMe
Robinin	740.2	H	OH	H	ORob	ORha
Rutin	610.2	OH	OH	H	ORut	OH

Figure I1.5.1 Structures of common flavonols and flavonol glycosides. Abbreviations: Ara, arabinose; Gal, galactose; Glu, glucose; Me, methyl; Rha, rhamnose; Rob, robinose; Rut, rutinose. Masses given are monoisotopic.

at the 3 position of the flavonol nucleus is the most common (Hollman and Arts, 2000). Glucose is the most prevalent sugar residue, although galactose, arabinose, xylose, and rhamnose are not unusual (Markham, 1982). Other mono-, di-, tri-, and tetrasaccharide derivatives, along with acylated and sulfated derivatives, are less frequently reported (Harborne and Williams, 2001). Altogether, more than 1200 flavonol glycosides have been identified to date (Harborne and Williams, 2001).

Traditionally, paper chromatography was used for the separation and identification of flavonol glycosides. However, in recent years, this methodology has essentially been replaced by high-performance liquid chromatography (HPLC; Merken and Beecher, 2000). Although HPLC is by far the most common method for identifying flavonol glycosides in a mixture, extensive purification prior to analysis may be required, and standards are not always available for definitive identification by comparison of retention times. In addition, separation of chemically similar compounds by chromatography can be laborious. Flavonol glycosides purified by HPLC are often further analyzed by nuclear magnetic resonance (NMR) to provide detailed structural information and confirm the identity. A number of mass spectrometry techniques has also been reported for analyzing flavonol glycosides, although extensive fragmentation, thermal instability, and requirement for derivatization can be prohibitive (Stobiecki, 2000).

MALDI-MS is a new method of flavonol glycoside analysis. The first published account was by Wang and Sporns (2000). Because of the large number of possible flavonol glycosides one could encounter in a sample, the inability of MALDI-MS to differentiate among isomers can be a liability. Definitive identification is best left to NMR analysis. However, MALDI-MS is useful as a rapid screening tool for preliminary identification of flavonol glycosides. Its main advantages include access to molecular weight information of parent ions, minimal sample preparation, and rapid analysis times. If standards are readily available, then HPLC analysis may be used in conjunction to confirm identities. In theory, flavonol glycosides from any type of plant material can be analyzed by MALDI-MS.

It is possible to quantify flavonol glycosides using an internal standard, but the process can

Table I1.5.1 Troubleshooting Guide for MALDI-MS Analysis of Flavonol Glycosides

Problem	Possible cause	Solution
No flavonol glycoside peaks	Co-extracted impurities may be suppressing the signal	Perform additional sample purification step (e.g., column chromatography[a])
	Nonuniform crystallization	Prepare sample in at least 30% aqueous solution
	Sample concentration too high	Dilute the sample
	Sample concentration too low	Concentrate the sample
	Matrix solution concentration too low	Concentrate matrix solution
Poor peak resolution	Laser power too high	Attenuate the laser power
	Detector voltage too high	Attenuate the detector voltage
Signal is very weak	Laser power too low	Increase laser power
	Detector voltage too low	Increase detector voltage
Observed mass does not match theoretical mass	Instrument is not calibrated	Calibrate the instrument using standards (slight variability, ~100 ppm, is normal)
High variability among identical runs	Nonuniform crystallization	Evaporate solvent as quickly as possible; prepare sample in at least 30% aqueous solution
	"Natural" variability	Collect several spectra per sample from different probe positions

[a]One way would be to use HPLC on a reversed-phase column using a gradient of acetonitrile and water with UV detection at 354 nm (Frison-Norrie and Sporns, 2002). Another alternative would be to perform a more selective extraction step using a series of organic solvents followed by column chromatography on silica gel or Sephadex LH 20 (Sang et al., 2002).

be quite complex. One must account for the multiple ions formed for each species, determine response ratios of the standard to all of the analytes, and account for fragmentation patterns, which can be tricky when some fragment ions and some parent ions are identical. For example, the appearance of quercetin glucoside in the mass spectrum may be due to loss of a rhamnose residue from quercetin rutinoside, or to natural occurrence in the sample. This is another reason to verify MALDI-MS results with another method, such as HPLC. For a more detailed discussion of quantification, refer to Frison-Norrie and Sporns (2002).

Critical Parameters

Flavonol glycosides may be non-uniformly distributed throughout the plant. For example, the seedcoats of nuts may be much richer in flavonol glycosides than the flesh. It may be beneficial in such circumstances to extract and analyze the flavonol glycoside-rich components separately to increase the concentration in the extract.

Preparation of the sample is crucial to the success of a MALDI-MS experiment. It is important to achieve a homogenous distribution of the analyte throughout the crystalline matrix structure in order to reduce the sample-to-sample and spot-to-spot variability. Homogeneity may be promoted by techniques such as rapid evaporation of the solvent under vacuum or "sandwich" layering techniques of matrix and analyte solutions, although often air-drying can be sufficient to achieve sufficient uniformity. Also, a partially aqueous sample solution (at least 30%) facilitates "beading" on the sample probe due to surface tension effects, which in turn leads to greater sample concentration and uniformity of the crystal bed. Generally, the matrix should be present in a 10^3 to 10^5 molar excess compared to the analyte.

Troubleshooting

See Table I1.5.1 for troubleshooting guidelines.

Anticipated Results

The highest response is generally observed for protonated ions (Fig. I1.5.2). Alkali cations—$[M+Na]^+$ and $[M+K]^+$—are of lower intensity and appear only for flavonol glycoside

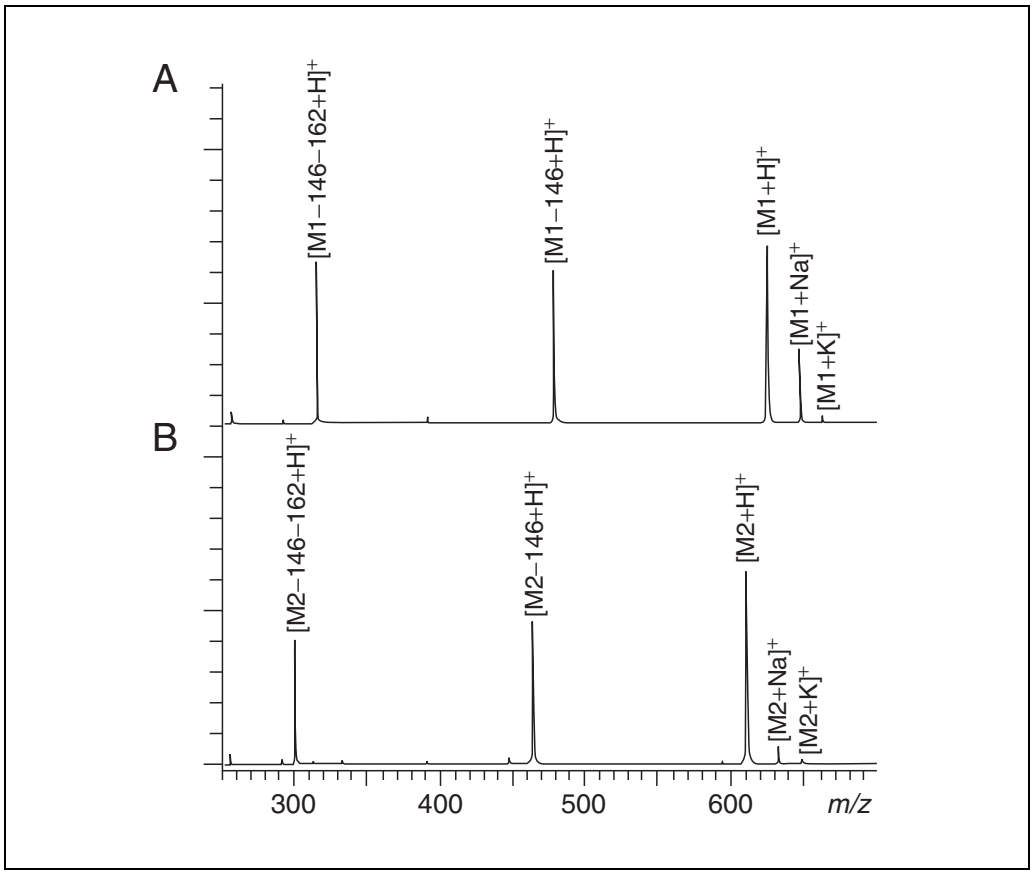


Figure I1.5.2 MALDI-MS natural cation spectra of flavonol glycoside standards. (**A**) M1= isorhamnetin-3-rutinoside (1.5×10^{-3} M in 70% methanol). (**B**) M2 = rutin (1.5×10^{-3} M in 70% methanol). Reprinted from Frison-Norrie and Sporns (2002) with permission from the American Chemical Society.

peaks and not for flavonol aglycone peaks. Larger molecules tend to have a greater preference for alkali adduct incorporation. When excess sodium is added to the extract, formation of $[M+K]^+$ ions will be suppressed, but $[M+2Na-H]^+$ ions may appear (Fig. I1.5.3). Consecutive loss of sugar residues creates characteristic fragment ions of variable intensity. The mass difference between parent ions and fragment ions can offer valuable structural information about the nature of the sugar residue.

Time Considerations

Preparation of the crude aqueous sample extract (Basic Protocol 1) requires 30 min for extraction, 15 min for gravity filtration (~2 min if vacuum filtration is used), and 30 to 40 min for rotary evaporation. This protocol can be completed in less than 2 hr.

Purification of the aqueous sample extract (Basic Protocol 2) requires ~5 min to condition the column, 5 min to load the sample, 5 to 10 min to wash, and 2 min to elute from the column. This protocol can be completed in ~20 min.

The rate-limiting step of MALDI-MS analysis (Basic Protocol 3) is the sample preparation, which involves depositing matrix and sample solutions onto the probe, allowing the solvent to evaporate, and introducing the probe into the ion source. This process may require up to 10 min per sample. From this point, analysis can be carried out in less than 1 min per sample. If multiple spectra are to be collected for each sample, accordingly more time will be required. Manipulation of spectra and peak labeling can be achieved in less than 5 min.

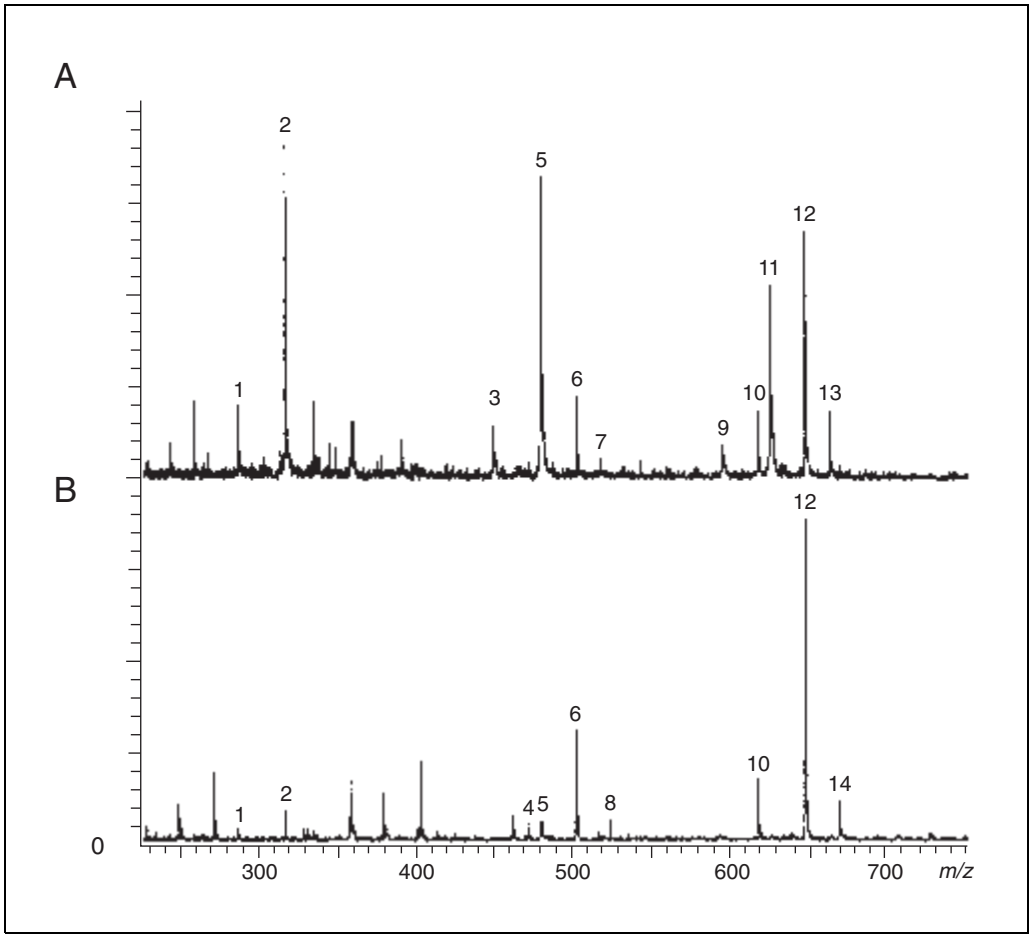


Figure I1.5.3 (**A**) Natural cation MALDI-MS spectrum of almond seedcoat extract. (**B**) MALDI-MS positive ion spectrum of almond seedcoat extract with sodium added at 0.01 M. Peaks: (1) $[kaempferol + H]^+$; (2) $[isorhamnetin + H]^+$; (3) $[kaempferol\ glucoside + H]^+$; (4) $[kaempferol\ glucoside + Na]^+$; (5) $[isorhamnetin\ glucoside + H]^+$; (6) $[isorhamnetin\ glucoside + Na]^+$; (7) $[isorhamnetin\ glucoside + K]^+$; (8) $[isorhamnetin\ glucoside + 2Na - H]^+$; (9) $[kaempferol\ rutinoside + H]^+$; (10) $[kaempferol\ rutinoside + Na]^+$; (11) $[isorhamnetin\ rutinoside + H]^+$; (12) $[isorhamnetin\ rutinoside + Na]^+$; (13) $[isorhamnetin\ rutinoside + K]^+$; (14) $[isorhamnetin\ rutinoside + 2Na - H]^+$.

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Key References

Frison, S. and Sporns, P. 2002. Variation in the flavonol glycosides composition of almond seedcoats as determined by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.* In press.

Describes use of MALDI-MS as a screening tool for flavonol glycosides in a selection of almond varieties.

Frison-Norrie and Sporns, 2002. See above.

Describes a MALDI-MS methodology for quantifying flavonol glycosides from almond seedcoats.

Wang and Sporns, 2000. See above.

First paper to describe a MALDI-MS methodology for identifying flavonol glycosides in foods.

Contributed by Suzanne Frison and Peter Sporns
University of Alberta
Edmonton, Alberta, Canada

Analysis of Isoflavones in Soy Foods

Isoflavones are characteristically found in the family of the Leguminosae, serving such biofunctions as phytoalexins and regulating growth and development of plants (Ingham, 1982). Increasing evidence suggests that consumption of soybean products may significantly impact health (Setchell, 1998, 2001; Birt et al., 2001). The biological activity has been associated, in part, with the presence of isoflavones in soy (Fournier et al., 1998; Setchell and Cassidy, 1999). Analysis of these bioactive compounds in soybean products is an essential part of any research involving soy isoflavones. This unit attempts to provide a reliable method with the most commonly used analytical techniques for this purpose.

Soybeans are the most prominent source of isoflavones in food plants, ranging in concentration from 0.1 to 3.0 mg/g dry weight (Coward et al., 1993). The general structure of isoflavones is shown in Figure I1.6.1, and the common names, chemical names, and chemical structures of the twelve isoflavones in soy are shown in Figure I1.6.2. Because isoflavones are present at small concentrations in soybeans and soy-based food products (see matrices in Table I1.6.1), their efficient extraction prior to analysis is very important. In this unit, extraction of the twelve soy isoflavones is described (see Basic Protocol 1), as is sample preparation before extraction (see Support Protocol 1). This process consists of removing water from the sample and sample grinding or homogenization.

When authentic isoflavone standards are available, the method of choice for analyzing isoflavone extracts from soy foods is generally high-performance liquid chromatography (HPLC) paired with a reversed-phase C18 column and UV spectrophotometer. A mixture of isoflavones in soy food extracts is separated based on the polarity and/or solubility of isoflavones in the column between the stationary (packing material of the column) and mobile (solvent) phases used. All isoflavones exhibit an intense absorption in the UV region of the spectrum, between 240 and 280 nm (Harborne, 1967). Based on the Beer-Lambert law, the concentration of a pure isoflavone compound or an individual isoflavone in a mixture can be calculated using the absorbance measurements obtained and known extinction coefficients (Ollis, 1962). This unit describes techniques for generating calibration curves with reference standards (see Basic Protocol 2), separating and measuring isoflavones using HPLC-UV spectrophotometry (see Basic Protocol 3), preparation of samples for HPLC (see Support Protocol 2), and converting glycosidic isoflavones to their aglycones using enzymatic hydrolysis (see Support Protocol 3).

NOTE: All reagents used in this unit, including water, should be HPLC grade or equivalent.

NOTE: Isoflavones should be protected from light, oxygen, and elevated temperature during and after extraction (see Critical Parameters and Troubleshooting).

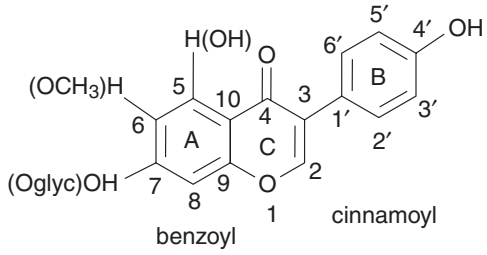


Figure I1.6.1 General structure and ring numbering system applied to known soy isoflavones.

Contributed by Yu Chu Zhang and Steven J. Schwartz

Current Protocols in Food Analytical Chemistry (2003) I1.6.1-I1.6.17

SOLVENT EXTRACTION OF ISOFLAVONES IN THEIR NATURAL FORMS FROM SOY FOODS

This method focuses on the extraction of soy food samples at room temperature using acidified solvent mixtures. The method is designed to release isoflavones in their natural forms from food matrices based on their polarity and solubility in solvents. Insoluble proteins, carbohydrates, and lipids present in the food are removed from the isoflavone extract using organic solvents, acid, and centrifugation.

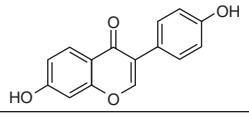
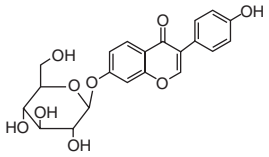
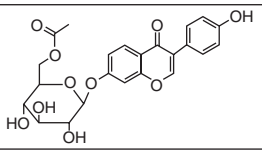
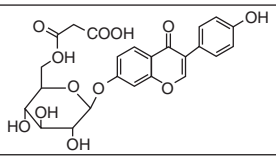
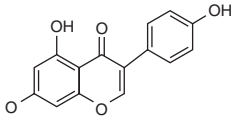
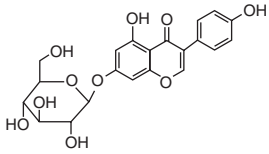
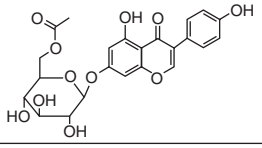
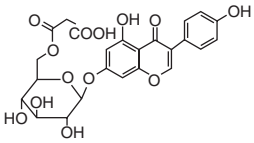
Common name	Chemical name	Chemical structure
Daidzein	7,4′-Dihydroxyisoflavone	
Daidzin	7,4′-Dihydroxyisoflavone 7-glucoside *or* daidzein 7-*O*-glucoside *or* daidzein 7-*O*-β-D-glucopyranoside	
Acetyldaidzin	6″-*O*-Acetyldaidzin	
Malonyldaidzin	6″-*O*-Malonyldaidzin	
Genistein	5,7,4′-Trihydroxyisoflavone *or* 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one	
Genistin	5,7,4′-Trihydroxyisoflavone 7-glucoside *or* genistein 7-*O*-glucoside *or* genistein-7-*O*-β-D-glucopyranoside	
Acetylgenistin	6″-*O*-Acetylgenistin	
Malonylgenistin	6″-*O*-Malonylgenistein	

Figure I1.6.2 *(above and at right)* Common name, chemical name, and chemical structure of soy isoflavones.

Materials

Prepared food sample (see Support Protocol 1)
Acetonitrile
0.1 M HCl

50-ml centrifuge tube with cap
Ultrasonic bath
Wrist shaker
15-ml centrifuge tube
Analytical pipets

1. In a 50-ml centrifuge tube with cap, carefully measure the prepared food sample and mix it with solvent (i.e., acetonitrile, HCl, and water) as detailed in Table I1.6.2. Break up cellular material using an ultrasonic bath for 10 min.
2. Vortex the mixture for 1 min to homogenize the suspension.
3. Extract on a wrist shaker 2 hr at room temperature (≤25°C).
4. Centrifuge the mixture 30 min at 430 × *g*, room temperature.
5. Transfer an aliquot from the centrifuge tube to a 15-ml test tube. Store the aliquot at 4°C prior to further purification for HPLC analysis.

   It is recommended that analysis of extracts be conducted within 10 hr of extraction to minimize potential conversion of isoflavones.

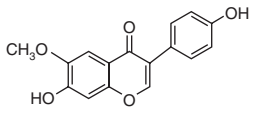
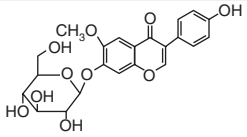
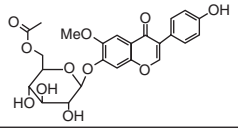
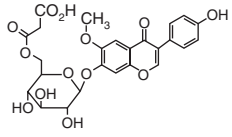
Glycitein	7,4′-Dihydroxy-6-methoxyisoflavone	
Glycitin	7,4′-Dihydroxy-6-methoxyisoflavone-7-D-glucoside	
Acetylglycitin	6″-*O*-Acetylglycitin	
Malonylglycitin	6″-*O*-Malonylglycitin	

Table I1.6.1 Matrices of Some Common Soybean Products and Their Isoflavone Content

	Protein (%[a])	Carbohydrate (%[a])	Lipids (%[a])	Isoflavones (mg/g on total basis[b])
Soybeans[c]	40	36	19	3.0
Full fat soy flour[d]	48	32	11	2.0
Defatted soy flour[e]	52	35	1	3.0
Soy milk powder[f]	40	32	18	2.8
Soy containing bread[g]	40	40	2	0.9

[a]Percent by weight on total basis.
[b]Milligrams/gram on total basis from company certificate of analysis unless specified.
[c]Harvested in Ohio.
[d]Expeller Soy Flour I.P. from SunRich.
[e]Defatted soy flour from Cargill Foods.
[f]Soy Supreme Basic Soy milk powder from SunRich.
[g]Soy-containing bread from Bavoy.

Table I1.6.2 Solvent Mixture for Extracting Isoflavones from Soy Foods[a]

	Sample	Acetonitrile	0.1 M HCl	Water
Dehydrated/solid sample	0.5 g	10 ml	2 ml	4 ml
Liquid sample	2 ml	10 ml	2 ml	2 ml

[a]Final mixture: ~60% acetonitrile/40% water.

Table I1.6.3 Commercial Sources of Soy Isoflavone Reference Standards[a]

Isoflavone	Apin Chemicals	Fisher Scientific	Indo Fine Chemical	LC Laboratories	Sigma Aldrich
Daidzein	X	X	X	X	X
Daidzin	X		X	X	X
Acetyldaidzin				X	
Malonyldaidzin				X	
Genistein	X	X	X	X	X
Genistin	X		X	X	X
Acetylgenistin				X	
Malonylgenistin				X	
Glycitein			X	X	X
Glycitin			X	X	X
Acetylglycitin				X	
Malonylglycitin				X	

[a]Ordering and product information can be found at the company websites (see *SUPPLIERS APPENDIX*).

SAMPLE PREPARATION FOR EXTRACTION

SUPPORT PROTOCOL 1

Extract fresh food samples whenever possible. Grind solid and/or dehydrated soybean products in a grinder or with a mortar and pestle until a fine powder (one that can pass through a 50-mesh screen) is obtained. Mash moist soybean product to a fine paste.

If food samples need to be stored for later extraction, seal in a plastic bag and store up to 10 days at –20°C or lower to prevent isoflavone loss and degradation. Thaw the sample completely at room temperature immediately before extraction. If freeze drying is desired, perform after food samples have been prefrozen in a freeze-dry flask to –20°C or lower.

PREPARATION OF INDIVIDUAL ISOFLAVONE STANDARDS

BASIC PROTOCOL 2

In this protocol, commercially purchased isoflavone standards (Table I1.6.3) are dissolved in a suitable solvent to prepare solutions in a series of decreasing concentrations. The absorbances are then measured using a UV spectrophotometer set at the isoflavone's maximum wavelength (λ_{max}), and the concentrations of isoflavone standard solutions are calculated using published molar extinction coefficients. The spectrum is also scanned in order to evaluate the fine structure (see Background Information, Spectral fine structure).

Materials

Standard isoflavones (see Support Protocol 3 and Tables I1.6.3 and I1.6.4)
80% (v/v) methanol

5-digit analytical balance
10- and 50-ml volumetric flasks
Ultrasonic bath
0.45-µm filter
Analytical pipets
Amber vials with caps
UV spectrophotometer and quartz/glass cuvette

Table I1.6.4 Isoflavone Stock Standard Solutions and Calibration Ranges

Isoflavones	Weight (mg) in 50 ml stock solution	Stock solution (mg/ml)	Working solutions (µg/ml)
Daidzein	2	0.04	0.4-8
Daidzin	5	0.1	1-20
Acetyldaidzin	2	0.04	0.4-8
Genistein	2	0.04	0.4-8
Genistin	5	0.1	1-20
Acetylgenistin	2	0.04	0.4-8
Glycitein	1	0.02	0.2-4
Glycitin	1	0.02	0.2-4
Acetylglycitin	1	0.02	0.2-4

Prepare stock standard solutions

1. Using a 5-digit analytical balance, weigh the amount of each crystalline isoflavone standard given in Table I1.6.4 and transfer to individual 50-ml volumetric flasks.
2. Add 80% methanol to near 50 ml, stopper each flask, and mix well by repeated inversion and by incubating in an ultrasonic bath until complete dissolution is achieved (~10 min).

   Dissolution may be aided by initial addition of a small amount of a more effective solvent (e.g., DMSO) before bringing to volume for spectrophotometric measurement (no more than 1% of the total volume should be adequate). Dissolution can also be aided by using warm solvent (≤50°C).

3. Fill each flask with 80% methanol to the final volume (50 ml). Seal flasks tightly with a cap and store up to 6 months at −20°C.
4. Before use, completely thaw frozen stock solutions at room temperature and then pass through a 0.45-µm filter.

Prepare working standard solutions

5. From each stock solution bottle, accurately transfer 0.1, 0.3, 0.5, 1, and 2 ml solution to 10-ml volumetric flasks using analytical pipets.
6. Dilute to volume using 80% methanol (see Table I1.6.4 for working concentrations). Mix well by repeated inversion of the flasks.
7. Transfer working solutions to tightly capped amber vials and store up to 2 months at −20°C. Completely thaw at room temperature before analysis.

   The working solutions will be used for UV absorbance measurement and for quantification by HPLC.Calibration should be performed at least every 2 months when new working standards are prepared.

Measure absorbance using a UV spectrophotometer

8. Turn on the UV spectrophotometer and allow to warm up at least 30 minutes.
9. Zero the spectrophotometer with an appropriate blank (i.e., 80% methanol).

Table I1.6.5 UV Spectral Pattern of Twelve Soy Isoflavones in 80:20 (v/v) Methanol/Water

Isoflavone	MW	Absorption peaks			λ_{max} (nm)	$\varepsilon_{\lambda max}$ (liter/mol·cm)[a]
Daidzein	254	211.4	249.1	303.6	249	31563
Daidzin	416	216.0	250.3	302.5	250	26830
Acetyldaidzin	458	216.1	249.1	301.5	249	29007
Malonyldaidzin	502	211.4	250.3	302.5	250	26830
Genistein	270	211.4	259.8	327.0	260	35323
Genistin	432	212.6	259.8	327.5	260	30895
Acetylgenistin	474	215.0	260.9	329.8	261	38946
Malonylgenistin	518	215.0	259.8	327.5	260	30895
Glycitein	284	215.0	257.4	321.5	257	25388
Glycitin	446	212.6	258.9	321.5	259	26713
Acetylglycitin	488	212.6	258.6	321.5	259	29595
Malonylglycitin	532	212.6	259.0	321.5	259	26313

[a]Values from Murphy et al. (2002).

10. Fill a quartz/glass cuvette with the standard solution of a specific isoflavone. Measure the absorbance at λ_{max} (see Table I1.6.5). Take reading immediately.

    With the dilution applied here, the absorbance reading should be in the range of 0 to 1.0.

11. Observe the UV spectrum (Table I1.6.5; also see Background Information, Spectral Fine Structure).

12. Repeat steps 9 to 11 for all standards and concentrations.

Calculate isoflavone concentration

13. Calculate the concentration of isoflavone standard solutions based on the Beer-Lambert law.

    $A = c$ (mol/liter) $\times$ l (cm) $\times$ ε (liter mol^{-1} cm^{-1})

    where A is absorbance, c is concentration, l is cell pathlength (typically 1 cm), and ε is the molar extinction coefficient (i.e., absorbance of a 1 M solution in a 1-cm light path at a specific wavelength; Table I1.6.5).

GRADIENT SEPARATION AND IDENTIFICATION OF ISOFLAVONES USING A C18 REVERSED-PHASE COLUMN

BASIC PROTOCOL 3

In this protocol, a reversed-phase separation using a C18 HPLC column and gradient mobile phase are used to separate isoflavones in the soy food extract. Reversed-phase HPLC operates on the basis of hydrophilicity and lipophilicity. Reversed-phase C18 columns consist of silica-based packings with covalently bound 18-alkyl chains. The mobile phase carries the sample solution through the column. The chemical and physical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components in the column. In gradient elution, different isoflavone compounds are separated by increasing the strength of the organic solvent. Detection is achieved by monitoring UV absorbance at 260 nm using a UV or photodiode array (PDA) detector. Retention time, UV spectra, and co-elution are used to identify individual isoflavones in food extracts by comparison with pure isoflavone compounds (see Basic Protocol 2).

Materials

Solvent A: 1% (v/v) acetic acid in water, pH >2.0, fresh
Solvent B: 100% acetonitrile
Isoflavone reference standards (see Basic Protocol 2)
Food sample: isoflavone extract from soy foods, after clean-up procedure (see Support Protocol 2)

Sonicator, vacuum filtration device, or in-line vacuum degasser
HPLC system:
- UV/Vis photodiode array (PDA) detector
- Pump: gradient
- Waters 2695 separation module with heating and cooling system
- Waters Nova-Pak C18 reversed-phase column (150 × 3.9 mm, 4-µm i.d., 60-Å pore size)
- Waters Nova-Pak C18 guard column or a guard column with similar packing material
- Injector: automatic
- Computer data system

Additional reagents and equipment for preparing calibration curves (see Basic Protocol 2)

Prepare mobile phase

1. Check pH of solvent A, making sure it is >2.0. Degas both mobile phases via vacuum filtration, ultrasonic agitation, or inline vacuum degasser.

   Acetic acid serves as a modifier to protonate glucosides of isoflavones in the mobile phase.

   While freshly prepared solvent A is preferred, solvent that is up to 2 days old is acceptable.

Set up HPLC apparatus

2. Turn on the UV/Vis PDA detector at least 30 minute before analysis.
3. Prime the gradient pump and flush the lines with mobile phases A and B, following manufacturer's instructions.
4. Program the mobile phase gradient as listed in Table I1.6.6.
5. Set the following HPLC parameters:

   Pump flow rate: 0.6 ml/min
   Column oven temperature: 25°C
   Injection volume: 10 µl
   UV monitor: 260 nm.

   For strong UV absorbers such as isoflavones, total injected amounts of <20 µg and injection volumes of 10 to 30 µl are typical. Larger injection volumes can cause broadening of peaks. The flow rate was developed based on the column inner diameter and mass sensitivity.

6. Equilibrate the system and column with the starting mobile phase composition (i.e., 85% A:15% B) of mobile phase A for 10 column volumes (10 × 1.8 ml/column volume), until the baseline is flat and smooth.

   The small particle size (4 µm) of the bonded phases and small pore size (60 Å) of the C18 column efficiently resolve mixtures of similar isoflavone derivatives in the soy food matrix.

Perform HPLC analysis

7. Inject each working calibration standard and standard mixture (including any optional internal standard) individually into the automatic injector.

   The standard mixture can be prepared by taking a known amount of each standard solution into a sample vial and mixing well. The concentration of each isoflavone in the standard mixture can be calculated with the dilution factor.

   To check the recovery of a chromatographic condition, it is common to spike a known concentration of standard solution into the sample solution. A matrix effect is suspected if the spike recovery is outside the limits of 90% to 110%. Recovery is calculated using the formula $R = [(C_s - C)/S] \times 100$, *where R is percent recovery,* C_s *is spiked sample concentration, C is sample background concentration, and S is concentration equivalent of spike added to sample. If a matrix effect is suspected, adjustment of chromatographic condition must take place.*

Table I1.6.6 Mobile Phase Gradients for Isoflavone Analysis Using a Reversed-Phase C18 Column

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15
5	85	15
36	71	29
44	65	35
45	85	15
50	85	15

8. Measure the peak area of each working standard solution at 260 nm. Record the retention time for each peak. Observe the UV spectrum of each peak.

   Modern HPLC systems commonly include computer software that automatically integrates peak areas. For older systems, manual integration may be necessary.

   Besides the retention time, the UV spectra of each peak should be used to confirm the identity of isoflavones (see Background Information, Spectral Fine Structure).

9. Inject food sample and record data as in step 8.

Analyze results

10. Prepare calibration curves by plotting peak area versus known concentration for each standard solution.

    The slope of the line will be used to calculate the concentration of the respective isoflavone.

    Peak area should be linearly proportional to analyte concentration ($y = ax + b$) with a correlation coefficient >0.98 and an intersect very near the origin. In this equation, y is the peak area, x is the concentration of standard solution, a is the response factor between peak area, and b is the concentration (~0).

11. Identify isoflavones in the food sample by comparing the retention times of peaks with co-elution of isoflavone standards. Confirm identification by comparing the spectra of isoflavones in the food sample with those of standards (see Background Information).

    The complete separation of all twelve isoflavones using this chromatographic condition requires ~30 min. The elution order is daidzin, glycitin, genistin, malonyldaidzin, malonylglycitin, acetyldaidzin, acetylglycitin, malonylgenistin, daidzein, glycitein, acetylgenistin, and genistein.

    Isoflavone retention and separation are influenced by column temperature. The oven temperature is set at 25°C.

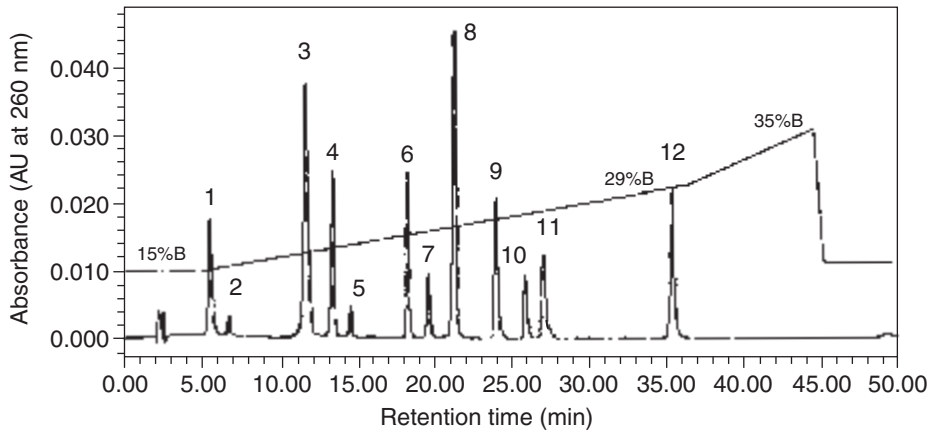


Figure I1.6.3 Gradient HPLC separation of isoflavone standards (see Basic Protocol 3). Peaks: 1, daidzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; 5, malonylglycitin; 6, acetyldaidzin; 7, acetylglycitin; 8, malonylgenistin; 9, daidzein; 10, glycitein; 11, acetylgenistin; 12, genistein. Conditions: Waters Nova-Pak C18 reversed-phase column (150 × 3.9 mm; 4-µm i.d.; 60 Å pore size); mobile phase: 1% acetic acid in water (solvent A) and acetonitrile (solvent B); flow rate: 0.60 ml/min; UV detector: 260 nm; column temperature: 25°C. The dotted line represents the gradient of solvent B.

12. Use the area of each isoflavone peak and the calibration curves to calculate the isoflavone concentrations in the sample. Calculate the final concentration of isoflavones in soy food by considering the dilution factor during sample preparation.

A sample chromatogram of isoflavone standards is shown in Figure I1.6.3.

SUPPORT PROTOCOL 2

SAMPLE PREPARATION FOR HPLC ANALYSIS

Soy foods are complex matrices (see Table I1.6.1). After initial aqueous extraction, the crude extract containing isoflavones is concentrated but may contain components that are immiscible with the HPLC mobile phase for direct injection. A clean-up step is necessary to prevent the introduction of particles and precipitates that may block the frit and foul the column.

Materials

Crude isoflavone extract (see Basic Protocol 1)
Methanol

10-ml glass tube with cap
Solvent evaporation apparatus (e.g., nitrogen gas, Speedvac)
Ultrasonic bath
0.20-μm filter

1. Transfer 1 ml supernatant from crude soy extract to a 10-ml glass tube. Dry the aliquot under a nitrogen gas stream or using a Speedvac evaporator.
2. Resuspend the residue in 1 ml of 100% methanol.
3. Ultrasonicate the mixture in a bath for 10 min.

   This step is designed for complete protein precipitation, removal of methanol-insoluble sugars, and isoflavone redissolution in solvent.

4. Store the mixture up to 10 hr at 4°C if injection is not directly conducted.
5. Before injection, vortex the mixture 1 min and filter through a 0.20-μm filter.

SUPPORT PROTOCOL 3

CONVERTING GLYCOSIDIC ISOFLAVONES TO THEIR AGLYCONES USING ENZYMES

Isoflavone aglycone standards are commercially available. However, the acetyl and malonyl glucosides of isoflavone standards are not readily available due to their lack of stability during transportation and storage. Hydrolysis of isoflavone glycosides removes the sugar moiety from the aglycones, thereby enabling quantification of total isoflavones in soy foods when the authentic standards of glycosidic isoflavones are not available. Hydrolysis is also a useful tool for structural analysis when specificity is achieved.

β-glucosidase (Emulsin) and cellulase (from *Aspergillus niger*) have been used efficiently to convert isoflavone conjugates to their aglycone forms (Franke et al., 1994; Liggins et al., 1998). They remove the β-linked glucose from the 7-hydroxyl group on the isoflavone aglycones with adequate purity. Figure I1.6.4 illustrates the method using enzymatic hydrolysis for isoflavone analysis.

By comparing the chromatogram from enzymatic hydrolysis with that in the absence of enzymatic hydrolysis, the completeness of hydrolysis by enzymes and the total isoflavones in soy food extract can be determined (see Basic Protocol 3).

Materials

Crude isoflavone extract (See Basic Protocol 1)
Enzyme: β-glucosidase, almond-isolate *or* cellulase, *Aspergillus niger* extract
0.1 M ammonium acetate buffer, pH 5 (adjust pH with acetic acid)
100% methanol

Solvent evaporation unit (nitrogen gas)
Ultrasonic bath
0.2-μm filter

1. Centrifuge the crude isoflavone extract 30 min at 430 × *g*, 25°C.
2. Take 1 ml supernatant from the crude extract and evaporate the solvent under nitrogen flow.
3. Dissolve 100 Fishman units of enzyme in 5 ml of 0.1 M ammonium acetate buffer, pH 5.

   One Fishman unit is the amount of enzyme necessary to digest 1 μmol substrate in 1 min at pH 5.0, 37°C.

4. Add 1 ml dissolved enzyme solution into the vial containing dried extract. Mix well in an ultrasonic bath for 10 min.
5. Incubate the solution 12 hr in a 37°C water bath.

   Overnight is recommended.

6. Evaporate the solvent under nitrogen flow.
7. Dilute the dried residue with 1 ml of 100% methanol. Vortex and/or place in an ultrasonic bath to ensure solubilization.
8. Pass the sample solution through a 0.2-μm filter immediately before HPLC analysis.

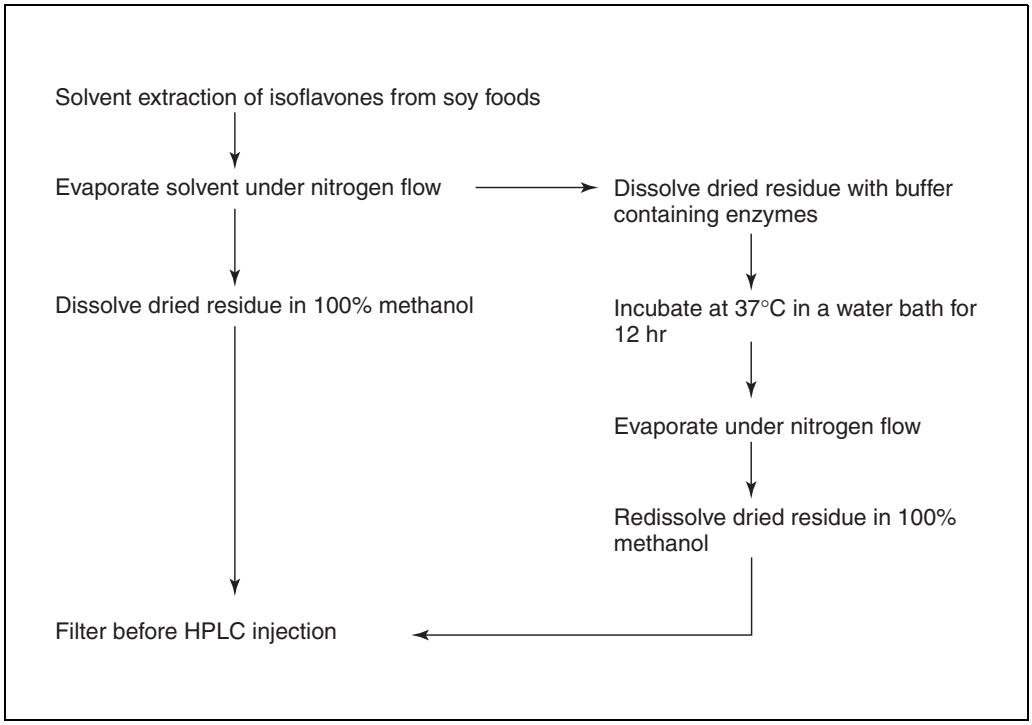


Figure I1.6.4 A flow diagram for isoflavone analysis using enzymatic hydrolysis.

COMMENTARY

Background Information

Isoflavones

Isoflavones comprise two benzene rings (A and B) linked through a heterocyclic pyrane C-ring at the 3 position (Fig. I1.6.1), which distinguishes them from flavones (Ingham, 1982; also see Figures I1.3.7 and I1.3.9 for comparison). The primary isoflavones in soybeans are the genistein, daidzein, and glycitein families (Fig. I1.6.2). Each family consists of its respective aglycone, β-glucoside, malonylglucoside, and acetylglucoside. Other isoflavones (i.e., formononetin or biochanin A) are present in only trace amounts (<1.0 μg/g).

Malonylglucosides are the predominant form of isoflavones in unprocessed soybeans. Previous studies have concluded that malonylglucosides are heat labile and easily converted to their corresponding acetylglucosides and/or β-glucosides depending on the thermal conditions of processing and preparation (Xu et al., 2002). Enzyme activity may be responsible for the formation of aglycones by hydrolysis of glucosides (Coward et al., 1998). Various processing conditions produce soy products with a wide range of isoflavone content and composition. Recent studies observed that the chemical forms and abundance of isoflavones in soy foods have a significant impact on their bioavailability and biological effects (King, 1998; Izumi et al., 2000; Setchell et al., 2002). It is thus very important to avoid altering the natural forms and abundance of the twelve soy isoflavones during extraction, identification, and quantification.

Extraction

Soybeans and soybean products contain high levels of protein, carbohydrates, and lipids (Table I1.6.1). As minor components of complex mixtures, isoflavones must first be separated from the bulk of the matrix constituents prior to analysis. Efficient extraction methods for isoflavones should account for their diverse structures, chemical properties, and the food matrix of which they are constituents. This unit describes a practical way of extracting isoflavones from soybean products in their natural forms using readily available solvents and laboratory equipment.

Before extracting a compound of interest from a complex food matrix, it is necessary to obtain knowledge about the physical and chemical nature of the sample—i.e., moisture content, stability in acid and base, and thermal stability. The hydrophobicity of the isoflavone forms is aglycone > acetylglucoside > malonylglucoside > β-glucoside, based on their chromatographic behavior on reversed-phase columns in the presence of an acid in the mobile phase to protonate the glycosidic isoflavones. The ester bonds of acetyl- and malonylglucose of isoflavones are labile at elevated temperatures and under acidic or basic conditions. The aqueous solubilities of the isoflavone aglycones are low and are pH dependent due to the acidic nature of their phenolic groups. Conju-

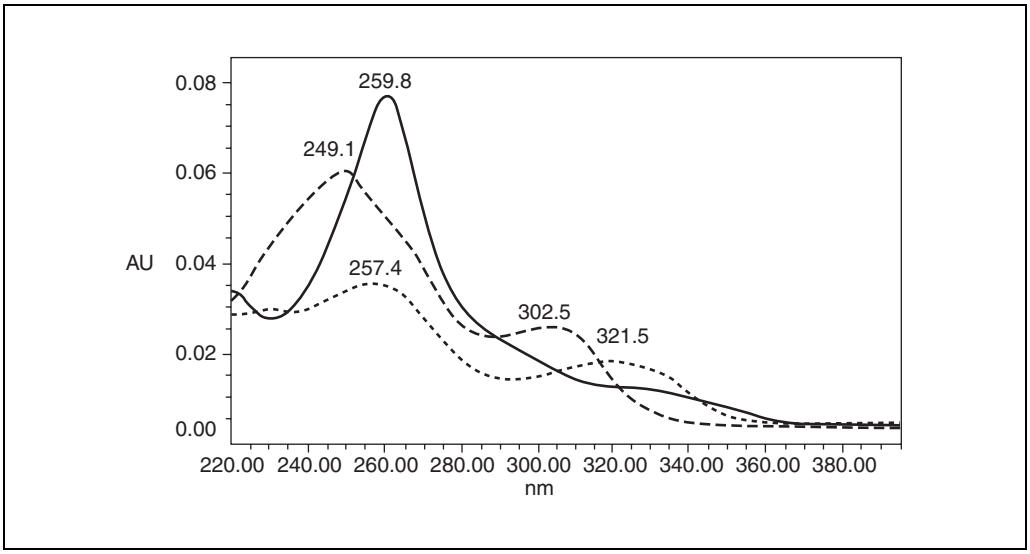


Figure I1.6.5 The UV characteristics of daidzein (dashed line), genistein (solid line), and glycitein (dotted line) in HPLC mobile phases (A: 1% acetic acid in water, B: 100% acetonitrile, A/B = 85:15) at 25°C monitored at 260 nm.

gation to glucose residues increases solubility, while acetylation or malonylation of the glucoses reduces solubility.

Spectral fine structure of soy isoflavones

The differences in the spectral characteristics of individual isoflavones are small but very important in their identification (Harborne, 1967). Isoflavones can be readily distinguished by their UV spectra, which typically exhibit an intense Band II (240 to 280 nm) absorption with only a shoulder or low-intensity peak representing Band I (300 to 330 nm). Band I absorption involves the B ring (cinnamoyl system; Figure I1.6.1), and Band II absorption involves the A ring (benzoyl system). The Band II absorption of isoflavones is relatively unaffected by increased hydroxylation of the B ring. Band II is, however, shifted bathochromically by increased oxygenation in the A ring. In addition to the absorption maxima of the isoflavones, the shape of the spectra provides important information for identification of pure isoflavone standards and isoflavones in soy extract. Fine structures of three soy isoflavone aglycones and their conjugates are shown in Figures I1.6.5 to I1.6.7. Details of the molecular characteristics of isoflavones can be found in *The Chemistry of Flavonoid Compounds* by Ollis (1962).

Isoflavones in solution obey the Beer-Lambert law, where absorbance (*A*) equals concen-

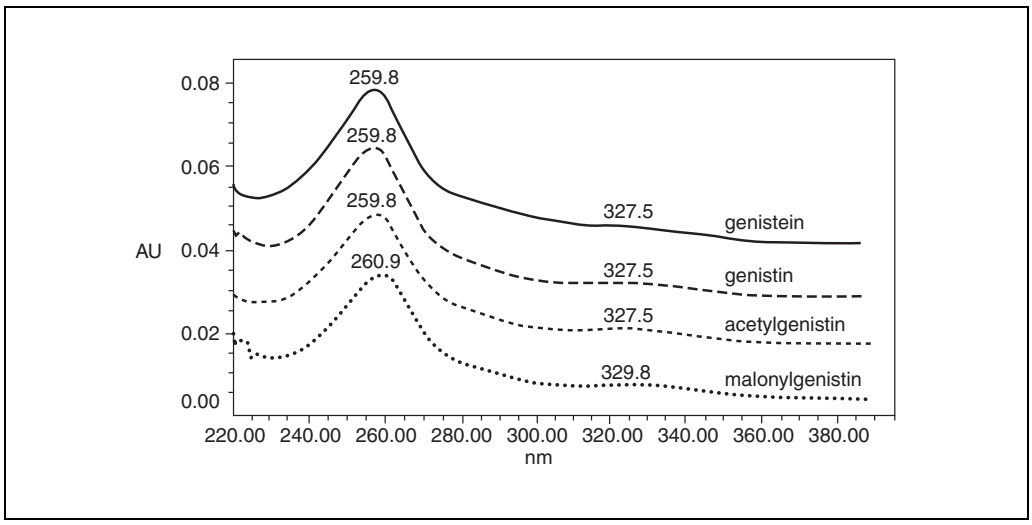


Figure I1.6.6 UV spectra of the genistein family in HPLC mobile phases (A: 1% acetic acid in water, B: 100% acetonitrile, A/B = 85:15) at 25°C, monitored at 220 to 400 nm.

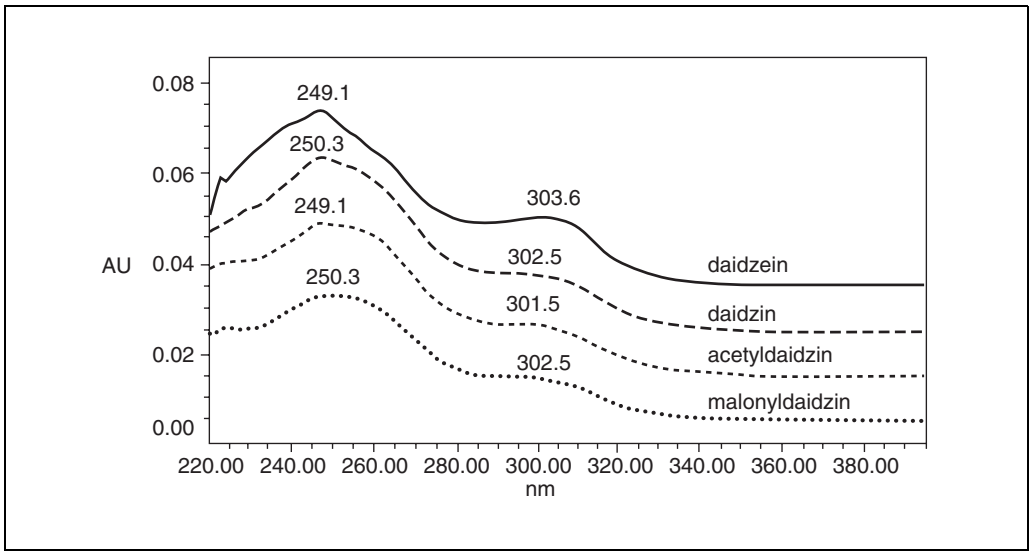


Figure I1.6.7 UV spectra of the daidzein family in HPLC mobile phases (A: 1% acetic acid in water, B: 100% acetonitrile, A/B = 85:15) at 25°C monitored at 220 to 400 nm.

tration multiplied by the molar extinction coefficient ($\varepsilon_{\lambda max}$). The molar extinction coefficient is defined as the absorbance of a 1 M solution of isoflavones, in a defined solvent, in a 1-cm-pathlength cuvette, at its maximum wavelength (λ_{max}). This information can be used to quantify the concentration of a certain isoflavone with known absorbance. Table I1.6.5 gives some known values of molar extinction coefficients of soy isoflavones. Variations in laboratory conditions (e.g., different solvents, temperature, absorbance wavelength) may be responsible for differences in reported extinction coefficients. The actual absorption and fine structure will depend on the composition of the mobile phase. A shift of 2 to 3 nm in the maximum absorption wavelength is usual. The spectrum of a specific isoflavone in soy food should be compared with an authentic pure standard. They should be identical for both the λ_{max} and the fine structure.

Isoflavone analysis using HPLC

HPLC is the method of choice for the analysis of isoflavones in soy products. HPLC is fast, reproducible, requires small sample sizes, and can be used for both qualitative and quantitative analysis as well as for separation purposes.

HPLC system. The basic HPLC system comprises a solvent delivery pump, injector, analytical column, and detector. Most isoflavone analyses are performed using a binary gradient, a revered-phase C18 column, and a UV detector. A photodiode array detector is capable of producing a UV/Vis absorption spectrum for each peak and has become the norm in isoflavone analysis. Isocratic elution was reported for isoflavone analysis but is not as effective as a gradient to separate the wide variety of isoflavones within an acceptable elution time. A typical gradient solvent used with reversed-phase C18 columns starts with a high proportion of polar solvents (i.e., water) and gradually increases the proportion of a less polar solvent (i.e., acetonitrile or methanol). The aqueous solvent is usually acidified to prevent ionization of isoflavone glycosides, which can give multiple peaks for some compounds.

Identification. The order of elution of isoflavones is largely independent of minor variations in the solvent system, and thus it is possible to make tentative identifications by comparing relative retention times and co-elution with pure isoflavone standards. The actual retention times will vary between different runs, usually within 1 min. Such variation could be caused by differences in mobile phase preparation and the HPLC system (i.e., oven temperature, column condition).

Quantification. For accurate quantification, a standard curve of peak area versus concentration should be constructed for each standard using the same chromatographic conditions (e.g., wavelength and solvent) as for the samples under analysis. The concentration range of standard curves should be determined according to both the isoflavone level of soy food samples and dilution factors during sample preparation such that the UV absorbance of the injected sample is within a range of 0 to 1. The appropriate standard curve can then be used to calculate the quantity of isoflavones represented by each HPLC peak in the sample.

Internal standards. The primary function of an internal standard is in the determination of the reliability of extraction, sample preparation, and chromatographic procedure. An internal standard can be added to the original sample. By comparing the peak area of the internal standard in the chromatogram with that of a control, the losses due to sample preparation can be established. Alternatively, an internal standard can be added to the extraction solvent or to the sample prior to HPLC injection to measure the recovery rate of the chromatographic system. A suitable candidate for an internal standard for isoflavone analysis should (1) be similar in structure to isoflavones but not present in soy foods, and (2) be completely separable from isoflavones by the same chromatographic conditions at a specific wavelength. Apigenin, 2,4,4′-trihydroxydeoxybenzoin (THB), flavone, and equilenin have been reportedly used as internal standards.

Hydrolysis

The hydrolytic removal of the carbohydrate component of isoflavone glycosides simplifies the quantitative analysis of the isoflavones. Besides enzymatic hydrolysis (described in Support Protocol 3), acid hydrolysis and alkaline hydrolysis are also reportedly used. Acid hydrolysis is used primarily for cleaving sugars from glycosides, while alkaline hydrolysis finds application in the specific removal of acyl groups from acylated glycosides to produce β-glucosides of isoflavones (Klump et al., 2001). However, these methods are less commonly used due to a variety of reasons, including incomplete hydrolysis of the glycosides and degradation of the unconjugated isoflavones (Liggins et al., 1998).

Critical Parameters and Troubleshooting

Extraction

The solvents used for extracting isoflavones from soy foods were chosen according to the solubility of isoflavones and the food matrix involved. The diversity of soy isoflavones in polarity requires the use of a combination of organic solvent and water for extraction. The organic-to-water ratio (10:5) was established based on Murphy's study on solvent selection (Murphy et al., 2002). Water content in the solvent needs to be adjusted according to the moisture content in soy foods. Freeze-drying of samples before extraction simplifies the extraction process, but is not a prerequisite.

Acetonitrile, acetone, ethanol, and methanol have been used to extract isoflavones from soy foods. Among them, acetonitrile proved to be the most efficient (Griffith et al., 2001; Murphy et al., 2002). The solvent is supplemented with 0.1 M HCl to completely un-ionize the isoflavones and to release them from protein complexes by denaturing and precipitating the proteins. Room temperature is recommended for extraction to avoid alteration of the natural forms of the isoflavones. The time for extraction, 2 hr, was chosen for maximum recovery and shortest processing time. Ultrasound is used to aid the extraction process by degrading and weakening the cellular matrix.

Isoflavones are relatively stable compounds, but can degrade under certain conditions. Daidzein and genistein are light sensitive. It is necessary to work in dim light and to avoid exposure of extracts to air. Sample tubes should be capped during extraction. It is recommended to wrap sample flasks with aluminum foil to avoid light. Malonylglycosides of isoflavones are heat labile. Samples should be kept in a refrigerator between preparation and analysis, or kept in a freezer (–20°C) for storage.

The crude extract needs to be further purified for HPLC analysis. Direct injection of the crude extract into the HPLC would clog the frit and analytical column with precipitated impurities (i.e., proteins).

Besides solvent extraction, supercritical carbon dioxide has been applied to extract isoflavones (Rostagno et al., 2002). Due to the hydrophobicity of carbon dioxide, supercritical fluid extraction is more suitable for extracting nonpolar aglycones than polar glycosides of isoflavones, and may not be quantitative.

Standard solutions

Follow good laboratory practices for UV spectrophotometry (*UNIT F2.2*). For the overall analysis, it is critical to prepare precise standard curves. Manufacturer's instructions and MSDS's should be considered to minimize hazards and avoid degradation prior to and during sample preparation. Pure isoflavone standards should be completely dissolved in solvent when preparing stock solutions. Ultrasound, addition of a small amount of a more effective solvent, and warming the solvent (up to 50°C) have proved to be efficient and safe methods for aiding dissolution of isoflavone standards without altering their original structure. Stock solutions should be kept at –20°C for storage purposes. The stock solution container needs to be sealed very well to avoid evaporation during storage. Stock solutions that have been stored frozen should be thawed at room temperature before reuse. Ultrasound is necessary to completely dissolve crystals that may develop during storage. All solutions should be filtered before reuse.

Sample preparation

Isoflavone glycosides can be deesterified and decarboxylated to their simpler conjugates under appropriate conditions. The conditions of sample handling and preparation prior to HPLC analysis are critical to ensure that there is no degradation or alteration of analytes. A handling temperature >60°C risks alteration of the original composition of isoflavones in the food matrix and should be avoided. Daylight or white fluorescent light should be avoided during sample preparation. Wrapping glass vials containing isoflavones in aluminum foil is suggested.

When resuspending isoflavones in pure organic solvent, extra care should be taken to completely dissolve extracted isoflavones in the solvent. Solublization can be achieved by ultrasonification and providing sufficient time for the isoflavones to dissolve. Vortexing helps to release and recover isoflavones from precipitated proteins that may stick to the wall of glass vials and containers. All solvents must be of a high degree of purity. All extracted samples should be filtered to remove small particles.

Hydrolysis

Quantitative results for different foods using the hydrolysis method need to be treated with caution. Hydrolysis conditions should be adjusted based on the knowledge of a crude soy food extract—i.e., the range of isoflavone con-

centration or the aglycone ratios. Partially hydrolyzed products lead to underestimation of the total isoflavones in the extracts.

HPLC analysis

Many parameters are critical to the successful and reproducible separation of isoflavones using HPLC.

Backpressure

Degassing should always be used to avoid outgassing and air bubble formation during HPLC analysis, especially when using a gradient. Backpressure can be used as an indicator for the condition of the column and the system. An abnormally high backpressure can be a sign of a fouled guard column. An abnormally low backpressure can be a sign of a leak between tubing and fittings, which causes poor peak shape. Variations in backpressure during analysis should be <50 psi. Larger variations could be caused by a pump malfunction.

Column care

Column equilibration (~10 column vol. recommended) ensures baseline stability, good peak shape, and reproducible retention times. For the specific column chosen in this unit for isoflavone analysis, at least 18 ml total is needed to equilibrate the system with mobile phase. The common practice is to purge the pump system and connect the inlet end of the column to the injector outlet. The initial pump flow should be set at 0.1 ml/min and increased to 0.6 ml/min in 0.1 ml/min increments. Once a steady backpressure and baseline have been achieved, the column is ready to use. Before injecting samples, it is suggested to run a blank gradient first to clean the column and help check for the possibility of impurity peaks.

For overnight storage, the column should be kept flushing at 0.1 ml/min with a mild solvent (i.e., acetonitrile or methanol). This practice reduces the re-equilibration time the following day to a few minutes rather than an hour or more. For long-term storage, the column should be flushed with its shipping solvent (i.e., acetonitrile for a Waters Nova-Pak RP-C18 column) at 1 ml/min for at least 15 min before being taken off and tightly sealed at both ends.

When contamination collects on a column, poor peak shapes (usually tailing) and extraneous peaks will appear with an increase in backpressure. Therefore, samples should always be filtered before injection. Mobile phases should be filtered a few days after preparation to remove any particles and prevent microbial growth. Nevertheless, after repeated use, the column needs to be cleaned or regenerated, because tightly bound impurities will bind to the stationary phase and reduce the attraction the column packing has for the sample components, thus reducing the retention times for the peaks of interest as the column ages. The manufacturer's instructions should be followed to regenerate the column. To prolong the life of expensive analytical columns, it is recommended that a filter and a guard column be placed before the analytical column to serve as a protective factor.

Solvent preparation

To prepare mobile phases, clean HPLC-grade solvent must be used along with clean solvent bottles. Since most of the organic solvents are volatile, they should be well sealed in solvent bottles after preparation. Acetic acid is subject to evaporation, causing the pH of the aqueous acetic acid solution to shift. Freshly prepared acetic acid in water is recommended for each set of analyses. The calculated pH of 1% acetic acid in water is ~2.7; however, it is suggested to check the pH of the acid solution each time it is prepared to assure its pH is >2.

Anticipated Results

The extraction protocol typically recovers 96% to 108% of isoflavones. The HPLC protocol typically recovers isoflavones in a range between 97% and 105% when tested using spiked reference standards. During reversed-phase HPLC separation, all isoflavones elute from the column in the order of polar to nonpolar and are separated within 50 min of run time.

Time Considerations

When freeze-drying is not included, completion of the full extraction process requires ~1/2 day. It is recommended that freeze-drying, which requires at least 12 hr, be performed overnight before the day of extraction.

Calibration prior to HPLC requires 1 or 2 days to perform all necessary steps (i.e., prepare stock solutions, filter, measure absorbance, dilute, and HPLC injection of the working solutions). Once the stock solution and working solutions have been prepared, a calibration check can be performed on a UV spectrophotometer within 1 hr. The sample clean-up procedure for HPLC analysis may require a few hours. Enzymatic hydrolysis requires overnight incubation. Each HPLC analysis requires 50 min. When an autosampler is available,

samples can be injected and analyzed overnight. Typically, one technician can extract 12 samples per day to be analyzed automatically overnight.

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ABBREVIATIONS AND USEFUL DATA

APPENDIX 1

Abbreviations Used in This Manual

APPENDIX 1A

AACC American Association of Cereal Chemists
ACS American Chemical Society
AED atomic emission detection
AEDA aroma extract dilution analysis
AMC 7-amido-4-methylcoumarin
ANS anilinonapththalene sulfonate
AOAC Association of Official Analytical Chemists
AOCS American Oil Chemists' Society
AOM active oxygen method
AOS allene oxide synthase
AP alkaline phosphatase
APCI atmospheric pressure chemical ionization
ASTM American Society for Testing and Materials
ATR attenuated total reflection
AU absorbance units
AV acid value
BCA bicinchoninic acid
BET Brunauer-Emmet-Teller (equation)
BGG bovine gamma globulin
BHA butylated hydroxyanisole
BHC branched hydrocarbons
BHT butylated hydroxytoluene
Brij 35 polyoxyethylene 23-lauren ether
°**Brix** measure of sugar content as determined by refractometer with a Brix scale
BSA bovine serum albumin
BV biological value
CAPT compensated attached proton test
CD conjugated diene; circular dichroism
CDTA *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid
CETAB cetyltrimethylammonium bromide
Chl *a* and *b* chlorophyll *a* and *b*
CI chemical ionization
CID collision-induced dissociation
CIE Commission Internationale de l'Éclairage (International Commission for Illumination)
CI/MS chemical ionization/mass spectrometry
CLA conjugated linoleic acids
CLSM confocal laser-scanning microscopy
CMC critical micelle concentration
COSY correlation spectroscopy
CP-HPLC chiral-phase high-performance liquid chromatography
CPA *cis*-parinaric acid
CT conjugated triene
CV coefficient of variation
cyd cyanidin
DAD diode array detector
DCI direct exposure chemical ionization
DCM dichloromethane
DEI direct exposure electron impact
Deoxy Mb deoxymyoglobin
DH degree of hydrolysis
DHP dihydroxy pigment
DMF dimethylformamide
DMSO dimethylsulfoxide
DNPH 2,4-dinitrophenylhydrazine
DOPA 3,4-dihydroxyphenylalanine
dpd delphinidin
DPO diphenol oxidase
DQF-COSY double quantum filtered correlation spectroscopy
DSA drop shape analysis
DSC differential scanning calorimetry
DTNB 5,5′-dithiobis(2-nitrobenzoic acid)
DTT dithiothreitol
DVT drop volume tensiometer
EC Enzyme Commission
EDTA ethylenediaminetetraacetic acid
EI electron impact
EI/MS electron impact/mass spectrometry
ELSD evaporative light-scattering detector
EM expressible moisture
EPA (U.S.) Environmental Protection Agency
ERH equilibrium relative humidity
ESI electrospray ionization
FAB/MS fast atom bombardment mass spectrometry
FAME fatty acid methyl ester
FC Folin-Ciocalteau
FDA (U.S.) Food and Drug Administration
FFA free fatty acids
FID free induction decay; flame ionization detection
FOX ferrous oxidation/xylenol orange method
FP fecal protein
FPD flame photometric detection
FPLC fast protein liquid chromatography
FTIR Fourier-transform infrared (spectrometry)

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g gravity (in expressions of relative centrifugal force)
GAB Guggenheim-Anderson-DeBoer (equation)
GC gas chromatography
GC/FID gas-liquid chromatography with flame ionization detection
GC/MS gas-liquid chromatography with mass selective detection
GC/O gas chromatography/olfactometry
GLC gas-liquid chromatography
GOPOD glucose oxidase/peroxidase (reagent)
HBS hydroxybenzenesulfonamide
HDPE high-density polyethylene
HEC hydroxyethylcellulose
HEPES *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]
HIC hydrophobic-interaction chromatography
HMBC heteronuclear multiple bond correlation
HMDS hexamethyldisilazane
HPLC high-performance liquid chromatography
HRGC high -resolution gas chromatography
HRP horseradish peroxidase
HS headspace
HS-SPME headspace solid-phase microextraction
HSQC heteronuclear single quantum coherence
i.d. inner diameter
IDA isotope dilution assay
IEF isoelectric focusing
IgG immunoglobulin G
IPA indole-3-propionic acid
IRMS isotope ratio mass spectrometry
IS internal standard
ISO International Standard Organization
IUB International Union of Biochemistry
IUBMB International Union of Biochemistry and Molecular Biology
IUPAC International Union of Pure and Applied Chemistry
IV iodine value
K_M Michaelis constant
Kat Katal (catalytic unit for enzyme activity)
LC-APCI-MS liquid chromatography/atmospheric pressure chemical ionization mass spectrometry
LED light-emitting diode
LOX lipoxygenase
LSIMS liquid secondary ion mass spectrometry
LVE linear viscoelastic region
MA malonaldehyde; malondialdehyde
MALDI matrix-assisted laser desorption/ionization
MALDI-TOF MS matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MCAC metal-chelate affinity chromatography
MCC microcrystalline cellulose
MDGC multidimensional gas chromatography
2-ME 2-mercaptoethanol
Me HODES methyl hydroxyoctadecadienoates
MES 2-(*N*-morpholino)ethanesulfonic acid
MetMb metmyoglobin
MFP metabolic fecal protein
MHP monohydroxy pigment
MOPS 3-(*N*-morpholino)propane sulfonic acid
MS mass spectrometry; mass selective (detection)
MS/MS tandem mass spectrometry
mvd malvidin
MWCO molecular weight cutoff
m/z mass-to-charge ratio
NBS National Bureau of Standards
NIST National Institute of Standards and Technology
NMR nuclear magnetic resonance
NO-heme nitrosylheme
NOESY nuclear Overhäuser enhancement spectroscopy
NPLC normal-phase HPLC
NPR net protein ratio
NPU net protein utilization
OAV odor activity value
o.d. outer diameter
OSI oil stability index
OU odor units
Oxy Mb oxymyoglobin
PBS phosphate-buffered saline
PDA photodiode array
PDCAAS protein digestibility–corrected amino acid score
PDMS polydimethylsiloxane
PE pectinesterase
PEG polyethylene glycol
PER protein efficiency ratio
PGase polygalacturonase
pgd pelargonidin
pI isoelectric point
PIPES piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)
PL pectic lyase
PMSF phenylmethylsulfonyl fluoride

p-NA paranitroanilide
pnd peonidin
psi pounds per square inch
ptd petunidin
PTFE polytetrafluoroethylene
PUFA polyunsaturated fatty acid
PV peroxide value
PVDF polyvinylidene difluoride
PVP polyvinylpyrrolidone
PVPP polyvinylpolypyrolidone
RAS retronasal aroma stimulator
RDA recommended dietary allowance
RF radio frequency
RFI relative fluorescence intensity
RI retention index
RNU relative nitrogen utilization
ROESY rotational nuclear Overhäuser enhancement spectroscopy
RP-HPLC reversed-phase HPLC
RPER relative protein efficiency ratio
RS resistant starch
RT retention time
RVP relative vapor pressure
S sieman (unit of conductance)
SD standard deviation
SDE simultaneous distillation extraction
SDS sodium dodecyl sulfate
SFC solid fat content
SFI solid fat index
SHAM salicylhydroxamic acid
SIM selected ion monitoring
SNIF-NMR site-specific natural isotope fractionation measured by nuclear magnetic resonance spectroscopy
SP-HPLC straight-phase high-performance liquid chromatography
SPME solid-phase microextraction
SV saponification value
TA titratable acidity
TBA thiobarbituric acid
TBARS thiobarbituric acid-reactive substances
TBS Tris-buffered saline
TCA trichloracetic acid
TD true digestibility
TEA triethylamine
TFA trifluoroacetic acid
THF tetrahydrofuran
TLC thin-layer chromatography
TLCK *N*α-*p*-tosyl-L-lysine chloromethyl ketone
TMCS trimethylchlorosilane imidazole
TMG tetramethylguanidine
TMP 1,1,3,3-tetramethoxypropane
TMS trimethylsilyl
TNBS trinitrobenzenesulfonic acid
TOCSY total correlation spectroscopy
TPA texture profile analysis
TPCK *N*-tosyl-L-phenylalanine chloromethyl ketone
TRF theoretical relative response factor
Tris tris(hydroxymethyl)aminomethane
Tris·Cl Tris hydrochloride
TTS time-temperature superposition
U unit (of enzyme activity)
UHP ultra high purity
USDA United States Department of Agriculture
UV ultraviolet
WHC water holding capacity
WUA water uptake ability

LABORATORY STOCK SOLUTIONS, EQUIPMENT, AND GUIDELINES

Common Buffers and Stock Solutions

This section describes the preparation of buffers and reagents used in the manipulation of nucleic acids.

For preparation of acid and base stock solutions, see Tables A.2A.1 and A.2A.2 as well as individual recipes.

GENERAL GUIDELINES

When preparing solutions, use deionized, distilled water and (for most applications) reagents of the highest grade available. Sterilization is recommended for most applications and is generally accomplished by autoclaving. Materials with components that are volatile, altered or damaged by heat, or whose pH or concentration are critical should be sterilized by filtration through a 0.22-μm filter. In many cases such components are added from concentrated stocks after the solution has been autoclaved. Where specialized sterilization methods are required, this is indicated in the individual recipes.

CAUTION: It is important to follow laboratory safety guidelines and heed manufacturers' precautions when working with hazardous chemicals; consult institutional safety officers and appropriate references for further details.

STORAGE

Most simple stock solutions can be stored indefinitely at room temperature if reasonable care is exercised to keep them sterile; where more rigorous conditions are required, this is indicated in the individual recipes.

Table A.2A.1 Molarities and Specific Gravities of Concentrated Acids and Bases[a]

Acid/base	Molecular weight	% by weight	Molarity (approx.)	1 M solution (ml/liter)	Specific gravity
Acids					
Acetic acid (glacial)	60.05	99.6	17.4	57.5	1.05
Formic acid	46.03	90	23.6	42.4	1.205
		98	25.9	38.5	1.22
Hydrochloric acid	36.46	36	11.6	85.9	1.18
Nitric acid	63.01	70	15.7	63.7	1.42
Perchloric acid	100.46	60	9.2	108.8	1.54
		72	12.2	82.1	1.70
Phosphoric acid	98.00	85	14.7	67.8	1.70
Sulfuric acid	98.07	98	18.3	54.5	1.835
Bases					
Ammonium hydroxide	35.0	28	14.8	67.6	0.90
Potassium hydroxide	56.11	45	11.6	82.2	1.447
Potassium hydroxide	56.11	50	13.4	74.6	1.51
Sodium hydroxide	40.0	50	19.1	52.4	1.53

[a]*CAUTION:* Handle strong acids and bases carefully.

Table A.2A.2 pK_a Values and Molecular Weights for Some Common Biological Buffers[a]

Name	Chemical formula or IUPAC name	pK_a	Useful pH range	Mol. wt. (g/mol)
Phosphoric acid	H_3PO_4	2.12 (pK_{a1})	—	98.00
Citric acid[b]	$C_6H_8O_7$ (H_3Cit)	3.06 (pK_{a1})	—	192.1
Formic acid	HCOOH	3.75	—	46.03
Succinic acid	$C_4H_6O_4$	4.19 (pK_{a1})	—	118.1
Citric acid[b]	$C_6H_7O_7^-$ (H_2Cit^-)	4.74 (pK_{a2})	—	
Acetic acid	CH_3COOH	4.75	—	60.05
Citric acid[b]	$C_6H_6O_7^-$ ($HCit^{2-}$)	5.40 (pK_{a3})	—	
Succinic acid	$C_4H_5O_4^-$	5.57 (pK_{a2})	—	
MES	2-(*N*-Morpholino]ethanesulfonic acid	6.15	5.5-6.7	195.2
Bis-Tris	bis(2-Hydroxyethyl)iminotris (hydroxymethyl)methane	6.50	5.8-7.2	209.2
ADA	*N*-(2-Acetamido)-2-iminodiacetic acid	6.60	6.0-7.2	190.2
PIPES	Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)	6.80	6.1-7.5	302.4
ACES	*N*-(Carbamoylmethyl)-2-amino-ethanesulfonic acid	6.80	6.1-7.5	182.2
Imidazole	1,3-Diaza-2,4-cyclopentadiene	7.00	—	68.08
Diethylmalonic acid	$C_7H_{12}O_4$	7.20	—	160.2
MOPS	3-(*N*-Morpholino)propanesulfonic acid	7.20	6.5-7.9	209.3
Sodium phosphate, monobasic	NaH_2PO_4	7.21 (pK_{a2})	—	120.0
Potassium phosphate, monobasic	KH_2PO_4	7.21 (pK_{a2})		136.1
TES	*N*-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid	7.40	6.8-8.2	229.3
HEPES	*N*-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)	7.55	6.8-8.2	238.3
HEPPSO	*N*-(2-Hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid)	7.80	7.1-8.5	268.3
Glycinamide HCl	$C_2H_6N_2O{\cdot}HCl$	8.10	7.4-8.8	110.6
Tricine	*N*-tris(Hydroxymethyl)methylglycine	8.15	7.4-8.8	179.2
Glycylglycine	$C_4H_8N_2O_3$	8.20	7.5-8.9	132.1
Tris	Tris(hydroxymethyl)aminomethane	8.30	7.0-9.0	121.1
Bicine	*N*,*N*-bis(2-Hydroxyethyl)glycine	8.35	7.6-9.0	163.2
Boric acid	H_3BO_3	9.24	—	61.83
CHES	2-(*N*-Cyclohexylamino)ethane-sulfonic acid	9.50	8.6-10.0	207.3
CAPS	3-(Cyclohexylamino)-1-propane-sulfonic acid	10.40	9.7-11.1	221.3
Sodium phosphate, dibasic	Na_2HPO_4	12.32 (pK_{a3})	—	142.0
Potassium phosphate, dibasic	K_2HPO_4	12.32 (pK_{a3})	—	174.2

[a]Some data reproduced from *Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems* (Mohan, 1997) with permission of Calbiochem.
[b]Available as a variety of salts, e.g., ammonium, lithium, sodium.

SELECTION OF BUFFERS

Table A.2A.2 reports pK_a values for some common buffers. Note that polybasic buffers, such as phosphoric acid and citric acid, have more than one useful pK_a value. When choosing a buffer, select a buffer material with a pK_a close to the desired working pH (at the desired concentration and temperature for use). In general, effective buffers have a range of approximately 2 pH units centered about the pK_a value. Ideally the dissociation constant—and therefore the pH—should not shift with a change in concentration or temperature. If the shift is small, as for MES and HEPES, then a concentrated stock solution can be prepared and diluted without adjustment to the pH. Buffers containing phosphate or citrate, however, show a significant shift in pH with concentration change, and Tris buffers show a large change in pH with temperature. For convenience, concentrated stock solutions of these buffers can still be used, provided that a pH adjustment is made *after* any temperature and concentration adjustments. All adjustments to pH should be made using the appropriate base—usually NaOH or KOH, depending on the corresponding free counterion. Tetramethylammonium hydroxide can be used to prepare buffers without a mineral cation. Many common buffers are supplied both as a free acid or base and as the corresponding salt. By mixing precalculated amounts of each, a series of buffers with varying pH values can conveniently be prepared.

RECIPES

Ammonium acetate, 10 M

Dissolve 385.4 g ammonium acetate in 150 ml H_2O
Add H_2O to 500 ml
Sterilize by filtration

Citrate-phosphate buffer (McIlvaine's buffer)

Solution A: 19.21 g/liter citric acid (0.1 M final)
Solution B: 53.65 g/liter $Na_2HPO_4 \cdot 7H_2O$ *or* 71.7 g/liter $Na_2HPO_4 \cdot 12H_2O$

Referring to Table A.2A.3 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize, if necessary, using a 0.2 µm filter and store up to 1 month 4°C.

DTT (dithiothreitol), 1 M

Dissolve 1.55 g DTT in 10 ml water and filter sterilize. Store in aliquots at −20°C.

Do not autoclave to sterilize.

EDTA (ethylenediaminetetraacetic acid), 0.5 M (pH 8.0)

Dissolve 186.1 g disodium EDTA dihydrate in 700 ml water. Adjust pH to 8.0 with 10 M NaOH (~50 ml; add slowly). Add water to 1 liter and filter sterilize.

Begin titrating before the sample is completely dissolved. EDTA, even in the disodium salt form, is difficult to dissolve at this concentration unless the pH is increased to between 7 and 8. Heating the solution may also help to dissolve EDTA.

HCl, 1 M

Mix in the following order:
913.8 ml H_2O
86.2 ml concentrated HCl (Table A.2A.1)

KCl, 1 M

74.6 g KCl
H_2O to 1 liter

$MgCl_2$, 1 M

20.3 g $MgCl_2 \cdot 6H_2O$
H_2O to 100 ml

$MgCl_2$ is extremely hygroscopic. Do not store opened bottles for long periods of time.

$MgSO_4$, 1 M

24.6 g $MgSO_4 \cdot 7H_2O$
H_2O to 100 ml

NaCl, 5 M

292 g NaCl
H_2O to 1 liter

NaOH, 10 M

Dissolve 400 g NaOH in 450 ml H_2O
Add H_2O to 1 liter

Potassium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid per liter (0.2 M) in water.

Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$) per liter (0.2 M) in water.

Referring to Table A.2A.4 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.4, prepare closest higher pH, then titrate with solution A.

Table A.2A.3 Preparation of Citrate-Phosphate Buffers

Desired pH	Solution A (ml)	Solution B (ml)
2.6	44.6	5.4
2.8	42.2	7.8
3.0	39.8	10.2
3.2	37.7	12.3
3.4	35.9	14.1
3.6	33.9	16.1
3.8	32.3	17.7
4.0	30.7	19.3
4.2	29.4	20.6
4.4	27.8	22.2
4.6	26.7	23.3
4.8	25.2	24.8
5.0	24.3	25.7
5.2	23.3	26.7
5.4	22.2	27.8
5.6	21.0	29.0
5.8	19.7	30.3
6.0	17.9	32.1
6.2	16.9	33.1
6.4	15.4	34.6
6.6	13.6	36.4
6.8	9.1	40.9
7.0	6.5	43.6

[a]Adapted with permission from Fasman (1989).

Table A.2A.4 Preparation of 0.1 M Sodium and Potassium Acetate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

[a]Adapted by permission from CRC (1975).

Table A.2A.5 Preparation of 0.1 M Sodium and Potassium Phosphate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

[a]Adapted by permission from CRC (1975).

Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH_2PO_4 per liter (0.2 M final) in water.

Solution B: 34.8 g K_2HPO_4 per liter (0.2 M final) in water.

Referring to Table A.2A.5 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate simply by scaling up the amount of potassium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH of the concentrate by diluting an aliquot to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.5, prepare closest higher pH, then titrate with solution A.

SDS, 20% (w/v)

Dissolve 20 g SDS (sodium dodecyl sulfate or sodium lauryl sulfate) in water to 100 ml total volume with stirring. Filter sterilize using a 0.45-µm filter.

It may be necessary to heat the solution slightly to fully dissolve the powder.

Sodium acetate, 3 M

Dissolve 408 g sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in 800 ml H_2O
Adjust pH to 4.8, 5.0, or 5.2 (as desired) with 3 M acetic acid (see Table A.2A.1)
Add H_2O to 1 liter
Filter sterilize

Sodium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid per liter (0.2 M) in water.

Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) per liter (0.2 M) in water.

Referring to Table A.2A.4 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.4, prepare closest higher pH, then titrate with solution A.

Sodium phosphate buffer, 0.1 M

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ per liter (0.2 M final) in water.

Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ per liter (0.2 M) in water.

Referring to Table A.2A.5 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH by diluting an aliquot of the concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.5, prepare closest higher pH, then titrate with solution A.

Tris·Cl, 1 M

Dissolve 121 g Tris base in 800 ml H_2O
Adjust to desired pH with concentrated HCl
Adjust volume to 1 liter with H_2O
Filter sterilize if necessary
Store up to 6 months at 4°C or room temperature

Approximately 70 ml HCl is needed to achieve a pH 7.4 solution, and ~42 ml for a solution that is pH 8.0.

IMPORTANT NOTE: *The pH of Tris buffers changes significantly with temperature, decreasing approximately 0.028 pH units per 1°C. Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used. Because the pK_a of Tris is 8.08, Tris should not be used as a buffer below pH ~7.2 or above pH ~9.0.*

Always use high-quality Tris (lower-quality Tris can be recognized by its yellow appearance when dissolved).

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Laboratory Safety

Persons carrying out the protocols in the laboratory may encounter various hazardous or potentially hazardous materials including: radioactive substances; toxic chemicals and carcinogenic, mutagenic, or teratogenic reagents; and pathogenic and infectious biological agents. Most governments regulate the use of these materials; it is essential that they be used in strict accordance with local and national regulations. Cautionary notes are included in many instances throughout the manual, and some specific guidelines for working safely with chemicals are provided below (and references therein). However, we emphasize that users must proceed with the prudence and precautions associated with good laboratory practice, under the supervision of personnel responsible for implementing laboratory safety programs at their institutions and in compliance with designated guidelines of federal, state, and local officials.

HAZARDOUS CHEMICALS

It is not possible in the space available to list all the precautions to be taken when handling hazardous chemicals. Many texts have been written about laboratory safety; see Literature Cited for a selected list of examples. Obviously, all national and local laws should be obeyed as well as all institutional regulations. Controlled substances are regulated by the Drug Enforcement Administration. By law, Material Safety Data Sheets must be readily available. All laboratories should have a Chemical Hygiene Plan [29CFR Part 1910.1450] and institutional safety officers should be consulted as to its implementation. Help is (or should be) available from your institutional Safety Office. Use it.

Chemicals should be stored properly. For example, flammable chemicals (e.g., ethanol, methanol, acetone, methyl ethyl ketone, petroleum distillates, toluene, benzene, and other materials labeled flammable) should be stored in approved flammable storage cabinets, and flammable chemicals requiring refrigeration should be stored in explosion-proof refrigerators. Oxidizers should be segregated from other chemicals, and corrosive acids (e.g., sulfuric, hydrochloric, nitric, perchloric, and hydrofluoric acids) should also be stored in a separate cabinet, well-removed from the flammable organics.

Facilities should be appropriate for the handling of hazardous chemicals. In particular, hazardous chemicals should only be handled in chemical fume hoods, not in laminar flow cabinets. The functioning of these fume hoods should be periodically checked. Laboratories should also be equipped with safety showers and eye-washing facilities. Again, this equipment should be tested periodically to make sure that it functions correctly. Other safety equipment may be required depending on the nature of the materials being handled. In addition, researchers should be trained in the proper procedures for handling hazardous chemicals as well as other areas of laboratory operations, e.g., handling of compressed gases, use of cryogenic liquids, operation of high voltage power supplies, etc.

Before starting work, have a plan for dealing with spills or accidents; coming up with a good plan on the spur of the moment is difficult. For example, have the appropriate decontaminating or neutralizing agents prepared and close at hand. Small spills can probably be cleaned up by the researcher. In the case of larger spills, the area should be evacuated and help sought from those experienced and equipped for dealing with spills, e.g., your institutional safety department.

Protective equipment should include, at a minimum, eye protection, a lab coat, and gloves. Sandals, open-toed shoes, and shorts should not be worn. In certain circumstances other items of protective equipment may be necessary, e.g., a face shield. Different types of gloves exhibit different chemical resistance properties; listings of these properties are available (Forsberg and Keith, 1989). Gloves should, however, be regarded as the last line of defense and should be changed if they become contaminated, because many types of chemicals pass relatively freely through rubber. If possible, handling procedures should be designed so that gloves do not become contaminated. All common-sense precautions should be observed, e.g., do not pipet by mouth, keep unauthorized persons away from hazardous chemicals, prohibit eating and drinking in the lab, etc.

Order hazardous chemicals only in quantities that are likely to be used in a reasonable time. Buying large quantities at a lower unit cost is no bargain if someone (perhaps you) has to pay to dispose of surplus quantities. Substitute alcohol-filled thermometers for mercury-

Contributed by George Lunn
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filled thermometers. The latter are a hazardous chemical spill waiting to happen.

Although any number of chemicals commonly used in laboratories are toxic if used improperly, the toxic properties of a number of reagents require special attention. Many chemicals are considered carcinogenic, corrosive, flammable, lachrymatory, mutagenic, oxidizing, teratogenic, or toxic. Chemicals labeled carcinogenic range from those accepted by expert review groups as causing cancer in humans to those for which only minimal evidence of carcinogenicity exists. Oxidizers may react violently with oxidizable material, e.g., hydrocarbons, wood, and cellulose. Before using any chemical, thoroughly investigate all of its characteristics. Material Safety Data Sheets are readily available; they list some hazards but vary widely in quality. A number of texts describing hazardous properties are listed in Further Reading. In particular, Sax's Dangerous Properties of Industrial Materials, 8th ed. (Lewis, 1992) and Bretherick's Handbook of Reactive Chemical Hazards, 4th ed. (Bretherick, 1990) give comprehensive listings of known hazardous properties. However, these texts list only the known properties. Many chemicals have been tested only partially or not at all. Prudence dictates, therefore, that unless there is good reason for believing otherwise, all chemicals should be regarded as volatile, highly toxic, flammable human carcinogens and should be handled with care.

Waste should always be disposed of in accordance with all applicable regulations. Waste should be segregated according to institutional requirements, for example, into solid, aqueous, nonchlorinated organic, and chlorinated organic material. A collection (Lunn and Sansone, 1994) of techniques for the disposal of chemicals in laboratories has been published recently. Incorporation of these procedures into laboratory protocols can help to minimize waste disposal problems.

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Lunn, and Sansone, 1994. See above.

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Standard Laboratory Equipment

Special equipment is itemized in the materials list of each protocol. Listed below are standard pieces of equipment in the modern food science laboratory—i.e., items used extensively in this manual and thus not usually included in the individual materials lists. See *SUPPLIERS APPENDIX* for contact information for commercial vendors of laboratory equipment.

Applicators, cotton-tipped and wooden
Autoclave
Balances, analytical and preparative
Beakers
Biohazard disposal containers and bags
Blender (e.g., Waring Blendor)
Bottles, glass and plastic
Bunsen burners
Centrifuges, low-speed (6,000 rpm) and high-speed (20,000 rpm) refrigerated centrifuges, ultracentrifuge (20,000 to 80,000 rpm), and microcentrifuge that holds standard 0.5- and 1.5-ml microcentrifuge tubes
NOTE: *Centrifuge speeds are provided as g or as rpm (with example rotor models) throughout the manual.*
Cold room (4°C) or cold box
Computer (PC or Macintosh) and printer
Conical centrifuge tubes, 15- and 25-ml plastic
Cuvettes, plastic disposable, glass, and quartz
Darkroom and developing tank, or X-Omat automatic X-ray film developer (Kodak)
Desiccators (including vacuum desiccators) and desiccant
Dry ice
Filtration apparatus, for collecting acid precipitates on nitrocellulose filters or membranes
Flasks, glass (e.g., Erlenmeyer, beveled shaker)
Forceps
Freezers, −20° and −80°C
Gel electrophoresis equipment, horizontal full-size and minigel apparatus, vertical full-size and minigel apparatus for polyacrylamide protein gels, and specialized equipment for two-dimensional protein gels
Grinder (e.g., coffee grinder)
Heat-sealable plastic bags and apparatus
Heating blocks, thermostat-controlled metal heating block that holds test tubes and/or microcentrifuge tubes
Hoods, chemical and microbiological
Hot plates, with or without magnetic stirrer
Gloves, plastic and latex, disposable and asbestos
Graduated cylinders
Ice buckets
Ice maker
Immersion oil for microscopy
Kimwipes, or equivalent lint-free tissues
Lab coats
Laboratory glass ware
Light box, for viewing gels and autoradiograms
Liquid nitrogen and Dewar flask
Magnetic stirrers (with heater is useful)
Markers, including indelible markers and china-marking pencils
Microcentrifuge, Eppendorf-type, maximum speed 12,000 to 14,000 rpm
Microcentrifuge tubes, 1.5-ml and 0.5-ml
Microscope, standard optical model (optionally with epifluorescence or phase-contrast illumination)
Microscope slides and coverslips
Microwave oven, to melt agar and agarose
Mortar and pestle
Muffle furnace
Ovens, drying, vacuum, and microwave
Paper cutter, large size, for 46 × 57-cm Whatman paper sheets
Paper towels
Parafilm
Pasteur pipets and bulbs
pH meter and pH standard solutions
pH paper
Pipet bulbs, or battery-operated pipetting devices—e.g., Pipet-Aid (Drummond Scientific)
Pipets, Pasteur and graduated, glass and plastic, serological (1- to 25-ml)
Pipettors, adjustable delivery, volume ranges 0.5 to 10 µl, 10 to 200 µl, and 200 to 1000 µl
Plastic wrap, UV transparent (e.g., Saran Wrap)
Polaroid camera
Power supplies, 300-V for polyacrylamide gels; 2000- to 3000-V for some applications
Racks, for test tubes and microcentrifuge tubes
Radiation shield, Lucite or Plexiglas
Radioactive waste containers, for liquid and solid waste
Razor blades

Refrigerator, 4°C
Ring stands and rings
Rotator, end-over-end
Rubber bands
Rubber policemen
Rubber stoppers
Safety glasses
Scalpels and blades
Scintillation counter
Scissors
Shakers, orbital and platform
Spectrophotometer, UV and visible
Speedvac evaporator (Savant)
Stir-bars, assorted sizes
Tape, masking and electrician's
Thermometers
Timer
UV transilluminator
Vacuum aspirator
Vacuum line
Volumetric flasks
Vortex mixers
Wash bottles, plastic and glass
Water baths, variable temperature up to 80°C
Water purification equipment, e.g., Milli-Q system (Millipore) or equivalent
X-ray film cassettes and intensifying screens

Introduction to Mass Spectrometry for Food Chemistry

Almost a century ago, the first mass spectrometers were used to prove the existence of isotopes of the elements. During the first half of the 20th century, physicists and physical chemists used mass spectrometers to help characterize new elements and the fission products of radioactive elements as they were created or discovered. Other applications included the analysis of isotopic enrichment of elements and their inorganic derivatives. As this era of mass spectrometry reached maturity, by the 1940s, the analysis of organic molecules emerged as a new application of mass spectrometry. Beginning in 1945, organic mass spectrometers using electron impact (EI) ionization became commercially available and were used primarily by the petroleum industry. Toward the late 1950s, organic mass spectrometers began to be used for the analysis of a wider variety of organic molecules, and gradually became a fundamental analytical tool for the characterization of synthetic organic compounds.

During the 1960s, high-resolution, double-focusing magnetic sector instruments became available from multiple manufacturers and were widely used in organic chemistry for exact mass measurements and elemental composition analysis. EI was used for generating structurally significant fragment ions for compound identification, and rules for structure elucidation using mass spectrometry were developed (for a thorough review of EI and ion fragmentation pathways, see McLafferty and Turecek, 1993). Biomedical and food chemistry applications of mass spectrometry were developed during this time. Chemical ionization (CI), which was developed by researchers in the petroleum industry (Field, 1990), was quickly adopted as a softer ionization alternative to EI, useful in reducing fragmentation so that molecular weights could be confirmed more easily. CI became another standard ionization technique for mass spectrometry (see Figure A.3A.1 for a guide to the selection of ionization techniques in mass spectrometry).

GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

With the introduction of computerized data systems for data acquisition, reduction, and storage during the 1960s, the efficiency of mass spectrometric analysis grew rapidly and continues to grow to this day. The use of computers for data reduction and analysis helped gas chromatography/mass spectrometry (GC/MS) become a practical and powerful tool for qualita-

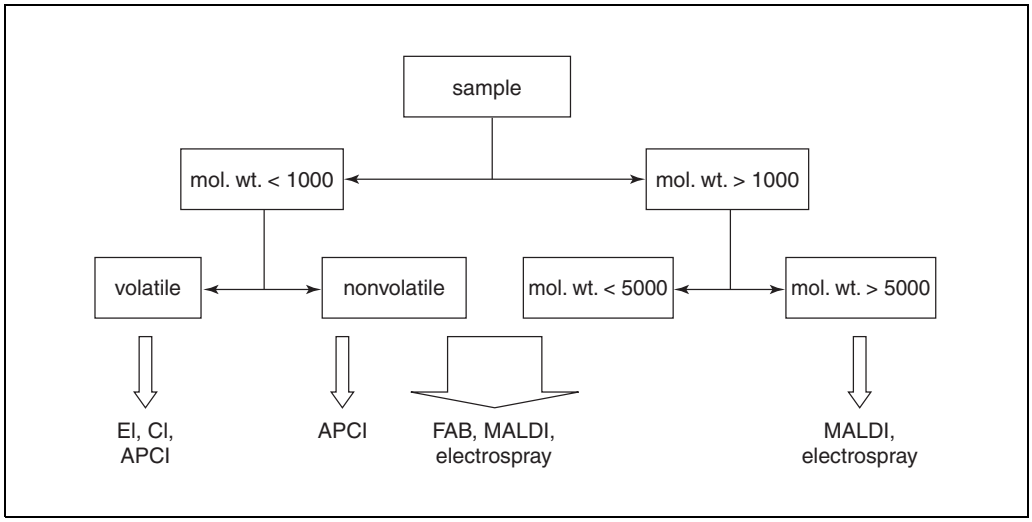

Figure A.3A.1 Flow chart illustrating the selection of a suitable ionization technique for the mass spectrometric analysis of a sample. Abbreviations: APCI, atmospheric pressure chemical ionization; CI, chemical ionization; EI, electron impact; FAB, fast atom bombardment; MALDI, matrix-assisted laser desorption/ionization.

Contributed by Richard B. van Breemen

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tive and quantitative analysis of compounds in mixtures. Both EI and CI were immediately useful for GC/MS, since both of these ionization methods require that the analytes be in the gas phase. When capillary GC was incorporated into GC/MS, this technique reached maturity. The advantages of GC/MS include speed, selectivity, and sensitivity. Typically, GC/MS may be used to select, identify, and quantify organic compounds in complex mixtures at the femtomole level. Compounds are selected using a combination of chromatographic separation and mass selection, and when using tandem mass spectrometry (MS/MS; see discussion below), the fragmentation pathway may be used for additional selectivity. The speed of GC/MS is determined by the chromatography step, which typically requires from several minutes to one hour per analysis. Although GC/MS remains important for the analysis of many organic compounds, this technique is limited to volatile and thermally stable compounds (see chromatography/MS selection flow chart in Fig. A.3A.2). Therefore, thermally unstable compounds—including food pigments such as carotenoids and chlorophylls and biomolecules such as proteins, carbohydrates, and nucleic acids—cannot be analyzed in their native forms using GC/MS (for more details regarding GC/MS and its applications, see Watson, 1997).

DESORPTION IONIZATION MASS SPECTROMETRY

During the 1970s and early 1980s, desorption ionization techniques such as field desorption (FD), desorption EI, desorption CI (DCI), and laser desorption were developed to extend the utility of mass spectrometry towards the analysis of more polar and less volatile compounds (see Watson, 1997, for more information regarding desorption ionization techniques including DCI and FD). Although these techniques helped extend the mass range of mass spectrometry beyond a traditional limit of *m/z* 1000 and toward ions of *m/z* 5000 (Fig. A.3A.1), the first breakthrough in the analysis of polar, nonvolatile compounds occurred in 1982 with the invention of fast atom bombardment (FAB; Barber et al., 1982). FAB and its counterpart, liquid secondary ion mass spectrometry (LSIMS), facilitate the formation of abundant molecular ions, protonated molecules, and deprotonated molecules of nonvolatile and thermally labile compounds such as peptides, chlorophylls, and complex lipids up to approximately *m/z* 12,000. FAB and LSIMS use energetic particle bombardment (fast atoms or ions from 3,000 to 20,000 V of energy) to ionize compounds dissolved in nonvolatile matrices such as glycerol or 3-nitrobenzyl alcohol and desorb them from this condensed phase into the gas phase for mass spectrometric analysis. Molecular ions and/or protonated molecules are usually abundant and fragmentation is minimal.

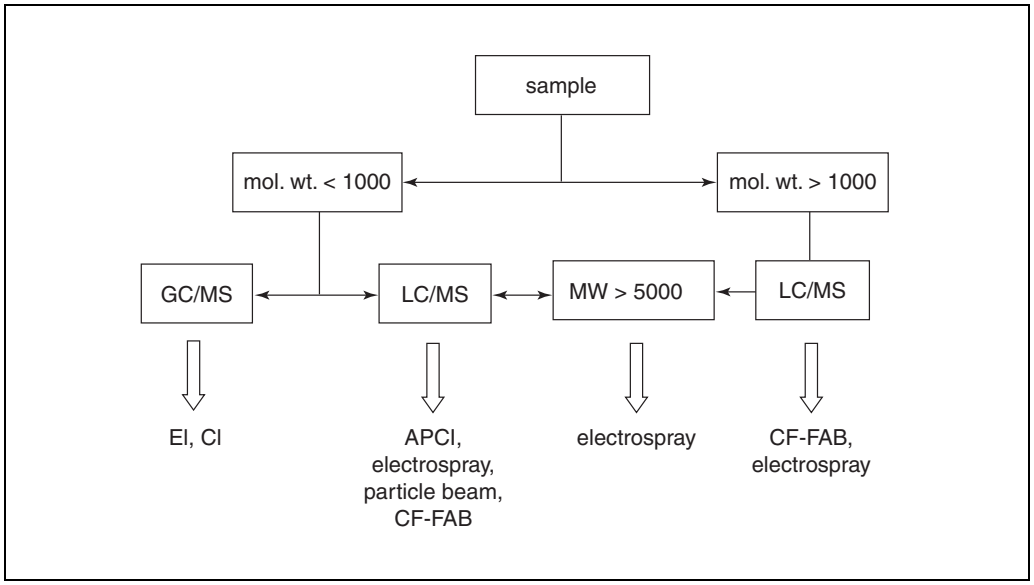

Figure A.3A.2 Selection of chromatography-mass spectrometry system for the analysis of a sample. Abbreviations: APCI, atmospheric pressure chemical ionization; CF, continuous flow; CI, chemical ionization; EI, electron impact; FAB, fast atom bombardment; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spectrometry.

Introduced in the late 1980s, matrix-assisted laser desorption/ionization (MALDI) has helped solve the mass-limit barriers of laser desorption mass spectrometry so that singly charged ions may be obtained up to *m/z* 500,000 and sometimes higher (Hillenkamp et al., 1991). For most commercially available MALDI mass spectrometers, ions up to *m/z* 200,000 are readily obtained. Like FAB and LSIMS, MALDI samples are mixed with a matrix to form a solution that is loaded onto the sample stage for analysis. Unlike the other matrix-mediated techniques, the solvent is evaporated prior to MALDI analysis, leaving sample molecules trapped in crystals of solid phase matrix. The MALDI matrix is selected to absorb the pulse of laser light directed at the sample. Most MALDI mass spectrometers are equipped with a pulsed UV laser, although IR lasers are available as an option on some commercial instruments. Therefore, matrices are often substituted benzenes or benzoic acids with strong UV absorption properties. During MALDI, the energy of the short but intense UV laser pulse obliterates the matrix and in the process desorbs and ionizes the sample. Like FAB and LSIMS, MALDI typically produces abundant protonated or deprotonated molecules with little fragmentation.

LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY (LC/MS)

By the time that GC/MS had become a standard technique in the late 1960s, LC/MS was still in the developmental stages. Producing gas-phase sample ions for analysis in a vacuum system while removing the HPLC mobile phase proved to be a challenging task. Early LC/MS techniques included a moving belt interface to desolvate and transport the HPLC eluate into a CI or EI ion source, or a direct inlet system in which the eluate was pumped at a low flow rate of 1 to 3 µl/min into a CI source. However, neither of these systems was robust enough or suitable for a broad enough range of samples to gain widespread acceptance.

Since FAB (or LSIMS) requires that the analyte be dissolved in a liquid matrix, this ionization technique was easily adapted for infusion of solution-phase samples into the FAB ionization source, in an approach known as continuous-flow FAB. Continuous-flow FAB was connected to microbore HPLC columns for LC/MS applications (Ito et al., 1985). Since this method is limited to microbore HPLC applications at flow rates of <10 µl/min and requires considerable operator intervention, it is not ideal for the analysis of large sample sets. Instead, more robust techniques have been developed to fulfill this requirement. However, continuous-flow FAB is still in use in some laboratories.

Like continuous-flow FAB, the popularity of particle beam interfaces is diminishing, but systems are still available from commercial sources. During particle beam LC/MS, the HPLC eluate is sprayed into a heated chamber connected to a vacuum pump. As the droplets evaporate, aggregates of analyte (particles) form and pass through a momentum separator that removes the lower-molecular-weight solvent molecules. Finally, the particle beam enters the mass spectrometer ion source where the aggregates strike a heated plate from which the analyte molecules evaporate and are ionized using conventional EI or CI ionization. Particle beam LC/MS is limited to the analysis of volatile and thermally stable compounds that are amenable to flash evaporation and EI or CI mass spectrometry. Therefore, this approach is not used for polar compounds in food chemistry such as carbohydrates, sugars, peptides, proteins, or nucleic acids (Fig. A.3A.2).

Since thermospray became the first widely utilized LC/MS technique (during the late 1970s and early 1980s), this technique should be mentioned here. Thermospray facilitates the interfacing of standard analytical HPLC systems at flow rates up to 1 ml/min with mass spectrometers. Although the interface between the HPLC and mass spectrometer is inefficient and exhibits low sensitivity for most analytes, thermospray has been useful for the LC/MS analysis of many types of small molecules. During thermospray, the HPLC eluate is sprayed through a heated capillary into a heated desolvation chamber at reduced pressure. Gas phase ions remaining after desolvation of the droplets are extracted through a skimmer into the mass spectrometer for analysis. The sensitivity of thermospray is poor since there is no mechanism or driving force to enhance the number of sample ions entering the gas phase from the spray during desolvation. Also, thermally labile compounds tend to decompose in the heated source. These problems were solved when thermospray was replaced by electrospray during the late 1980s.

During the 1990s, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) became the standard interfaces for LC/MS. Unlike thermospray, particle beam, or continuous-flow FAB, ESI and APCI inter-

faces operate at atmospheric pressure and do not depend upon vacuum pumps to remove solvent vapor. As a result, they are compatible with a wide range of HPLC flow rates. Also, no matrix is required. Both APCI and ESI are compatible with a wide range of HPLC columns and solvent systems. Like all LC/MS systems, the solvent system should contain only volatile solvents, buffers, or ion-pair agents, to reduce fouling of the mass spectrometer ion source. In general, APCI and ESI form abundant molecular ion species (Figures A.3A.1 and A.3A.2). When fragment ions are

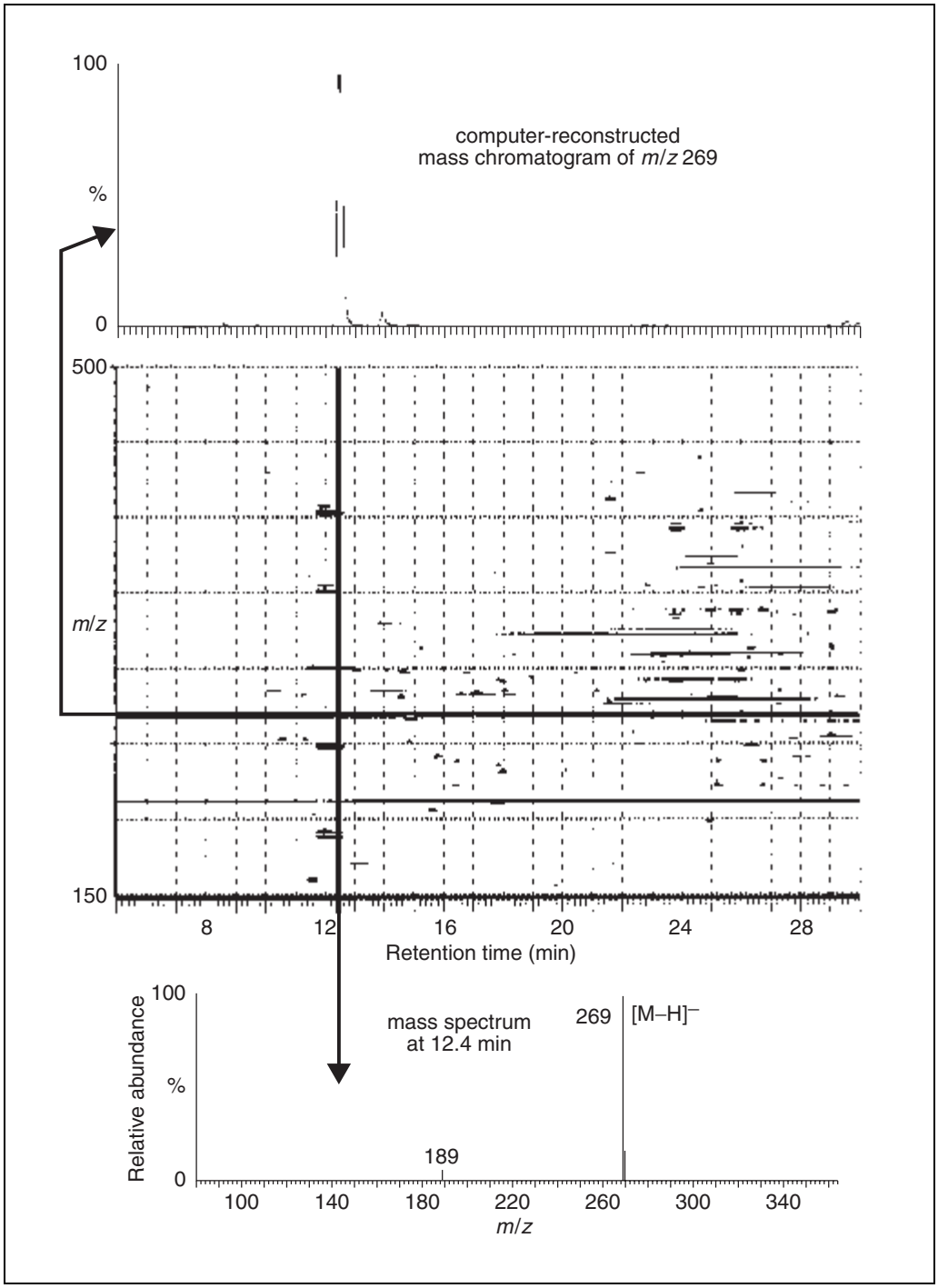

Figure A.3A.3 LC/MS analysis of a dietary supplement consisting of extract of *Trifolium pratense* (red clover). Reversed-phase C18 HPLC and negative ion electrospray ionization mass spectrometry were used with a quadrupole mass spectrometer analyzer (Agilent; also see Table A.3A.1). The map illustrates the abundance of information provided by this hyphenated technique with HPLC mass chromatograms in one dimension and mass spectra in another dimension.

formed, they are usually more abundant in APCI than ESI mass spectra.

The APCI interface uses a heated nebulizer to form a fine spray of the HPLC eluate, which is much finer than the particle beam system but similar to that formed during thermospray. A cross-flow of heated nitrogen gas is used to facilitate the evaporation of solvent from the droplets. The resulting gas-phase sample molecules are ionized by collisions with solvent ions, which are formed by a corona discharge in the atmospheric pressure chamber. Molecular ions, $M^{+\cdot}$ or $M^{-\cdot}$, and/or protonated or deprotonated molecules can be formed. The relative abundance of each type of ion depends upon the sample itself, the HPLC solvent, and the ion source parameters. Next, ions are drawn into the mass spectrometer analyzer for measurement through a narrow opening or skimmer, which helps the vacuum pumps to maintain very low pressure inside the analyzer while the APCI source remains at atmospheric pressure.

During ESI, the HPLC eluate is sprayed through a capillary electrode at high potential (usually 2000 to 7000 V) to form a fine mist of charged droplets at atmospheric pressure. As the charged droplets migrate towards the opening of the mass spectrometer due to electrostatic attraction, they encounter a cross-flow of heated nitrogen that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. Molecular ions, protonated or deprotonated molecules, and cationized species such as $[M+Na]^+$ and $[M+K]^+$ can be formed (for additional information on ESI, see Cole, 1997). In addition to singly charged ions, ESI is unique as an ionization technique in that multiply charged species are common and often constitute the majority of the sample ion abundance. The relative abundance of each of these species depends upon the chemistry of the analyte, the pH, the presence of proton-donating or -accepting species, and the levels of trace amounts of sodium or potassium salts in the mobile phase. In contrast, APCI, MALDI, EI, CI, and FAB/LSIMS usually produce singly charged species. A consequence of forming multiply charged ions is that they are detected at lower m/z values (i.e., $|z| > 1$) than the corresponding singly charged species. This has the benefit of allowing mass spectrometers with modest m/z ranges to detect and measure ions of molecules with very high masses. For example, ESI has been used to measure ions with molecular weights of hundreds of thousands or even millions of daltons on mass spectrometers with m/z ranges of only a few thousand (for a review of LC/MS techniques, see Niessen, 1999).

An example of the LC/MS analysis of a plant extract is shown in Figure A.3A.3. In this case, negative ion ESI-MS was used in combination with C18 reversed-phase HPLC separation. Extracts of the botanical *Trifolium pratense* (red clover) are used as dietary supplements by menopausal and post-menopausal women (Liu et al., 2001). The two-dimensional map illustrates the amount of information that may be

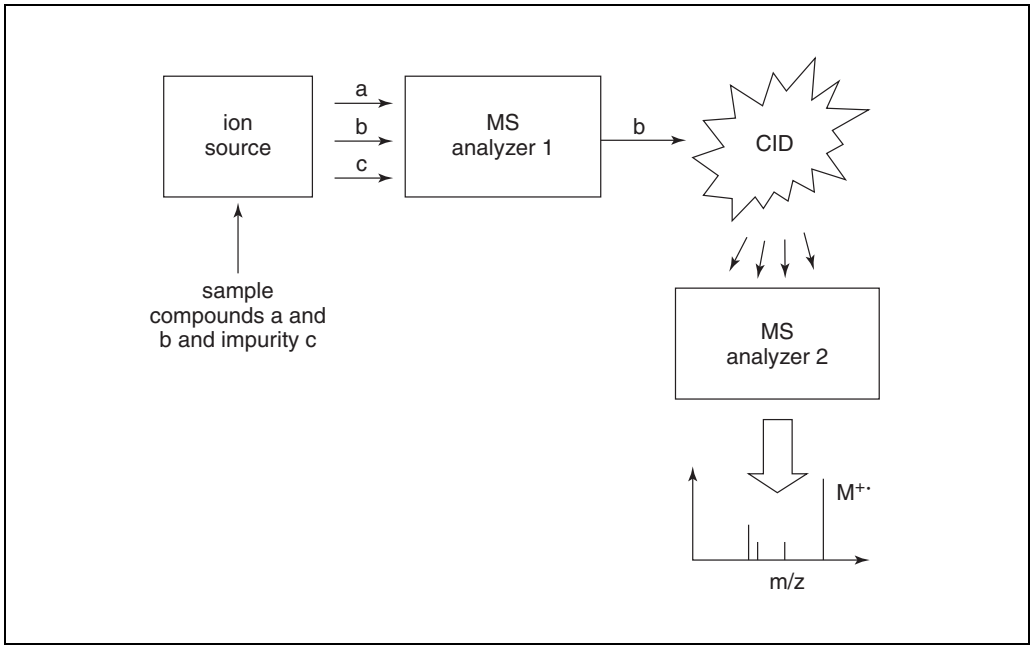

Figure A.3A.4 Scheme illustrating the selectivity of MS/MS and the process by which collision-induced dissociation (CID) facilitates fragmentation of preselected ions.

acquired using hyphenated techniques such as LC/MS. In the time dimension, chromatograms are obtained and a sample computer-reconstructed mass chromatogram is shown for the signal at *m/z* 269. One intense chromatographic peak was detected in this chromatogram eluting at 12.4 min. In the *m/z* dimension, the negative ion electrospray mass spectrum recorded at 12.4 min shows a base peak at *m/z* 269. Based on comparison to authentic standards (data not shown), the ion of *m/z* 269 was shown to correspond to the deprotonated molecule of genistein, which is an estrogenic isoflavone (Liu et al., 2001). Since almost no fragmentation of the genistein ion was observed, additional characterization would require collision-induced dissociation (CID) and tandem mass spectrometry as discussed in the next section.

TANDEM MASS SPECTROMETRY (MS/MS) AND HIGH RESOLUTION

Desorption ionization techniques like FAB and MALDI and LC/MS ionization techniques like ESI and APCI facilitate the molecular weight determination of a wide range of polar and nonpolar, low- and high-molecular-weight compounds. However, the "soft" ionization character of these techniques means that most of the ion current is concentrated in molecular ions and few structurally significant fragment ions are formed. In order to enhance the amount of structural information in these mass spectra, collision-induced dissociation (CID) may be used to produce abundant fragment ions from molecular ion precursors formed and isolated during the first stage of mass spectrometry. Then, a second mass spectrometry analysis may be used to characterize the resulting product ions. This process is called tandem mass spectrometry or MS/MS and is illustrated in Figure A.3A.4.

Another advantage of the use of tandem mass spectrometry is the ability to isolate a particular ion such as the molecular ion of the analyte of interest during the first mass spectrometry stage. This precursor ion is essentially purified in the gas phase and is free of impurities such as solvent ions, matrix ions, or other analytes. Finally, the selected ion is fragmented using CID and analyzed using a second mass spectrometry stage. In this manner, the resulting tandem mass spectrum contains exclusively analyte ions without impurities that might interfere with the interpretation of the fragmentation patterns. In summary, CID may be used with LC/MS/MS or desorption ionization and MS/MS to obtain structural information such as amino acid sequences of peptides and sites of alkylation of nucleic acids, or to distinguish structural isomers such as β-carotene and lycopene.

The most common types of MS/MS instruments available to researchers in food chemistry include triple quadrupole mass spectrometers and ion traps. Less common but commercially produced tandem mass spectrometers include magnetic sector instruments, Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, and quadrupole time-of-flight (QTOF) hybrid instruments (Table A.3A.1). Beginning in 2001, TOF-TOF tandem mass spectrometers became available from instrument manufacturers. These instruments have the potential to deliver high-resolution tandem mass spectra with high speed and should be compatible with the chip-based chromatography systems now under development.

In addition to MS/MS with CID to obtain structural information, it is also useful to use high-resolution exact mass measurements to confirm the elemental compositions of ions. Essentially, exact mass measurements permit the unambiguous composition analysis of low-molecular-weight compounds (mol. wt. <500) through precise and accurate *m/z* measurements. The types of mass spectrometers capable of exact mass measurements include magnetic sector mass spectrometers, QTOF hybrid

Table A.3A.1 Types of Mass Spectrometers and Tandem Mass Spectrometers[a]

Instrument	Resolution	*m/z* Range	Tandem MS
Magnetic sector	100,000	12,000	Low resolution
Quadrupole	<4,000	4,000	None
Triple quadrupole	<4,000	4,000	Low resolution
TOF	15,000	>200,000	None
FTICR	>200,000	<10,000	High resolution
QTOF	12,000	4,000	High resolution
TOF-TOF	15,000	>10,000	High resolution

[a]FTICR, Fourier transform ion cyclotron resonance; QTOF, quadropole time-of-flight; TOF, time-of-flight.

mass spectrometers, reflectron TOF instruments, and FTICR mass spectrometers (Table A.3A.1). Some of these instruments permit the simultaneous use of tandem mass spectrometry and exact mass measurement of fragment ions. These include FTICR instruments, QTOF, and the TOF-TOF.

CONCLUSION

Mass spectrometry has become an essential analytical tool for a wide variety of biomedical applications such as food chemistry and food analysis. Mass spectrometry is highly sensitive, fast, and selective. By combining mass spectrometry with HPLC, GC, or an additional stage of mass spectrometry (MS/MS), the selectivity increases considerably. As a result, mass spectrometry may be used for quantitative as well as qualitative analyses. In this manual, mass spectrometry is mentioned frequently, and extensive discussions of mass spectrometry appear, for example, in units describing the analyses of carotenoids (*UNIT F2.4*) and chlorophylls (*UNIT F4.5*). In particular, these units include examples of LC/MS and MS/MS and the use of various ionization methods.

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McLafferty and Turecek, 1993. See above.

This classic text describes fragmentation pathways and mechanisms for ions formed using electron impact (EI) ionization. In addition, this edition contains additional information regarding desorption ionization and the corresponding related fragmentation mechanisms.

Watson, 1997. See above.

This textbook provides an overview of biomedical mass spectrometry with particular emphasis on GC/MS and quantitative methods. In addition, descriptions are provided of the various types of mass spectrometers and ionization techniques that are used for biomedical applications.

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Chicago, Illinois

SELECTED SUPPLIERS OF REAGENTS AND EQUIPMENT

Listed below are addresses and phone numbers of commercial suppliers who have been recommended for particular items used in our manuals because: (1) the particular brand has actually been found to be of superior quality, or (2) the item is difficult to find in the marketplace. Consequently, this compilation may not include some important vendors of biological supplies. For comprehensive listings, see *Linscott's Directory of Immunological and Biological Reagents* (Santa Rosa, CA), *The Biotechnology Directory* (Stockton Press, New York), the annual Buyers' Guide supplement to the journal *Bio/Technology*, as well as various sites on the Internet.

A.C. Daniels
72-80 Akeman Street
Tring, Hertfordshire, HP23 6AJ, UK
(44) 1442 826881
FAX: (44) 1442 826880

A.D. Instruments
5111 Nations Crossing Road #8
Suite 2
Charlotte, NC 28217
(704) 522-8415 FAX: (704) 527-5005
http://www.us.endress.com

A.J. Buck
11407 Cronhill Drive
Owings Mill, MD 21117
(800) 638-8673 FAX: (410) 581-1809
(410) 581-1800
http://www.ajbuck.com

A.M. Systems
131 Business Park Loop
P.O. Box 850
Carlsborg, WA 98324
(800) 426-1306 FAX: (360) 683-3525
(360) 683-8300
http://www.a-msystems.com

Aaron Medical Industries
7100 30th Avenue North
St. Petersburg, FL 33710
(727) 384-2323 FAX: (727) 347-9144
http://www.aaronmed.com

Abbott Laboratories
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Abbott Park, IL 60064
(800) 323-9100 FAX: (847) 938-7424
http://www.abbott.com

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Lowell, MA 01852
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http://www.lomedco.com/abco.htm

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FAX: (44) 1970 615455
http://www.aber-instruments.co.uk

ABI Biotechnologies
See Perkin-Elmer

ABI Biotechnology
See Apotex

Access Technologies
Subsidiary of Norfolk Medical
7350 N. Ridgeway
Skokie, IL 60076
(877) 674-7131 FAX: (847) 674-7066
(847) 674-7131
http://www.norfolkaccess.com

Accurate Chemical and Scientific
300 Shames Drive
Westbury, NY 11590
(800) 645-6264 FAX: (516) 997-4948
(516) 333-2221
http://www.accuratechemical.com

AccuScan Instruments
5090 Trabue Road
Columbus, OH 43228
(800) 822-1344 FAX: (614) 878-3560
(614) 878-6644
http://www.accuscan-usa.com

AccuStandard
125 Market Street
New Haven, CT 06513
(800) 442-5290 FAX: (877) 786-5287
http://www.accustandard.com

Ace Glass
1430 NW Boulevard
Vineland, NJ 08360
(800) 223-4524 FAX: (800) 543-6752
(609) 692-3333

ACO Pacific
2604 Read Avenue
Belmont, CA 94002
(650) 595-8588 FAX: (650) 591-2891
http://www.acopacific.com

Acros Organic
See Fisher Scientific

Action Scientific
P.O. Box 1369
Carolina Beach, NC 28428
(910) 458-0401 FAX: (910) 458-0407

AD Instruments
1949 Landings Drive
Mountain View, CA 94043
(888) 965-6040 FAX: (650) 965-9293
(650) 965-9292
http://www.adinstruments.com

Adaptive Biosystems
15 Ribocon Way
Progress Park
Luton, Bedsfordshire LU4 9UR, UK
(44)1 582-597676
FAX: (44)1 582-581495
http://www.adaptive.co.uk

Adobe Systems
1585 Charleston Road
P.O. Box 7900
Mountain View, CA 94039
(800) 833-6687 FAX: (415) 961-3769
(415) 961-4400
http://www.adobe.com

Advanced Bioscience Resources
1516 Oak Street, Suite 303
Alameda, CA 94501
(510) 865-5872 FAX: (510) 865-4090

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9108 Guilford Road
Columbia, MD 21046
(800) 426-0764 FAX: (301) 497-9773
(301) 470-3220
http://www.abionline.com

Advanced ChemTech
5609 Fern Valley Road
Louisville, KY 40228
(502) 969-0000
http://www.peptide.com

Advanced Machining and Tooling
9850 Businesspark Avenue
San Diego, CA 92131
(858) 530-0751 FAX: (858) 530-0611
http://www.amtmfg.com

Advanced Magnetics
See PerSeptive Biosystems

Advanced Process Supply
See Naz-Dar-KC Chicago

Advanced Separation Technologies
37 Leslie Court
P.O. Box 297
Whippany, NJ 07981
(973) 428-9080 FAX: (973) 428-0152
http://www.astecusa.com

Advanced Targeting Systems
11175-A Flintkote Avenue
San Diego, CA 92121
(877) 889-2288 FAX: (858) 642-1989
(858) 642-1988
http://www.ATSbio.com

Advent Research Materials
Eynsham, Oxford OX29 4JA, UK
(44) 1865-884440
FAX: (44) 1865-84460
http://www.advent-rm.com

Advet
Industrivagen 24
S-972 54 Lulea, Sweden
(46) 0920-211887
FAX: (46) 0920-13773

Aesculap
1000 Gateway Boulevard
South San Francisco, CA 94080
(800) 282-9000
http://www.aesculap.com

Affinity Chromatography
307 Huntingdon Road
Girton, Cambridge CB3 OJX, UK
(44) 1223 277192
FAX: (44) 1223 277502
http://www.affinity-chrom.com

Affinity Sensors
See Labsystems Affinity Sensors

Affymetrix
3380 Central Expressway
Santa Clara, CA 95051
(408) 731-5000 FAX: (408) 481-0422
(800) 362-2447
http://www.affymetrix.com

Agar Scientific
66a Cambridge Road
Stansted CM24 8DA, UK
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FAX: (44) 1279-815-106
http://www.agarscientific.com

A/G Technology
101 Hampton Avenue
Needham, MA 02494
(800) AGT-2535 FAX: (781) 449-5786
(781) 449-5774
http://www.agtech.com

Agen Biomedical Limited
11 Durbell Street
P.O. Box 391
Acacia Ridge 4110
Brisbane, Australia
61-7-3370-6300 FAX: 61-7-3370-6370
http://www.agen.com

Agilent Technologies
395 Page Mill Road
P.O. Box 10395
Palo Alto, CA 94306
(650) 752-5000
http://www.agilent.com/chem

Agouron Pharmaceuticals
10350 N. Torrey Pines Road
La Jolla, CA 92037
(858) 622-3000 FAX: (858) 622-3298
http://www.agouron.com

Agracetus
8520 University Green
Middleton, WI 53562
(608) 836-7300 FAX: (608) 836-9710
http://www.monsanto.com

AIDS Research and Reference
Reagent Program
U.S. Department of Health and
Human Services
625 Lofstrand Lane
Rockville, MD 20850
(301) 340-0245 FAX: (301) 340-9245
http://www.aidsreagent.org

AIN Plastics
249 East Sanford Boulevard
P.O. Box 151
Mt. Vernon, NY 10550
(914) 668-6800 FAX: (914) 668-8820
http://www.tincna.com

Air Products and Chemicals
7201 Hamilton Boulevard
Allentown, PA 18195
(800) 345-3148 FAX: (610) 481-4381
(610) 481-6799
http://www.airproducts.com

ALA Scientific Instruments
1100 Shames Drive
Westbury, NY 11590
(516) 997-5780 FAX: (516) 997-0528
http://www.alascience.com

Aladin Enterprises
1255 23rd Avenue
San Francisco, CA 94122
(415) 468-0433 FAX: (415) 468-5607

Aladdin Systems
165 Westridge Drive
Watsonville, CA 95076
(831) 761-6200 FAX: (831) 761-6206
http://www.aladdinsys.com

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8561 154th Avenue NE
Redmond, WA 98052
(800) 543-2133 FAX: (425) 861-0173
(425) 882-2555
http://www.alcide.com

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Milwaukee, WI 53201
(800) 558-9160 FAX: (800) 962-9591
(414) 273-3850 FAX: (414) 273-4979
http://www.aldrich.sial.com

Alexis Biochemicals
6181 Cornerstone Court East, Suite 103
San Diego, CA 92121
(800) 900-0065 FAX: (858) 658-9224
(858) 658-0065
http://www.alexis-corp.com

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30 Bond Street
Ward Hill, MA 10835
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(978) 521-6300 FAX: (978) 521-6350
http://www.alfa.com

Alfa Laval
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BE-1140 Brussels, Belgium
32(2) 728 3811
FAX: 32(2) 728 3917 or 32(2) 728 3985
http://www.alfalaval.com

Alice King Chatham Medical Arts
11915-17 Inglewood Avenue
Hawthorne, CA 90250
(310) 970-1834 FAX: (310) 970-0121
(310) 970-1063

Allegiance Healthcare
800-964-5227
http://www.allegiance.net

Allelix Biopharmaceuticals
6850 Gorway Drive
Mississauga, Ontario
L4V 1V7 Canada
(905) 677-0831 FAX: (905) 677-9595
http://www.allelix.com

Allentown Caging Equipment
Route 526, P.O. Box 698
Allentown, NJ 08501
(800) 762-CAGE FAX: (609) 259-0449
(609) 259-7951
http://www.acecaging.com

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Applied Science Labs
2051 Waukegan Road
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(800) 255-8324 FAX: (847) 948-1078
(847) 948-8600
http://www.alltechweb.com

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http://www.alomone.com

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14743 Catalina Street
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(510) 483-9620
http://www.alphainnotech.com

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116 B Street
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(617) 269-1400

Alza
1900 Charleston Road
P.O. Box 7210
Mountain View, CA 94043
(800) 692-2990 FAX: (650) 564-7070
(650) 564-5000
http://www.alza.com

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P.O. Box 530
10240 Bubb Road
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FAX: (408) 865-1406
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(207) 854-0426

Amaresco
30175 Solon Industrial Parkway
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(440) 349-1313

Ambion
2130 Woodward Street, Suite 200
Austin, TX 78744
(800) 888-8804 FAX: (512) 651-0190
(512) 651-0200
http://www.ambion.com

American Association of
Blood Banks
College of American Pathologists
325 Waukegan Road
Northfield, IL 60093
(800) 323-4040 FAX: (847) 8166
(847) 832-7000
http://www.cap.org

American Bio-Technologies
See Intracel Corporation

American Bioanalytical
15 Erie Drive
Natick, MA 01760
(800) 443-0600 FAX: (508) 655-2754
(508) 655-4336
http://www.americanbio.com

American Cyanamid
P.O. Box 400
Princeton, NJ 08543
(609) 799-0400 FAX: (609) 275-3502
http://www.cyanamid.com

American HistoLabs
7605-F Airpark Road
Gaithersburg, MD 20879
(301) 330-1200 FAX: (301) 330-6059

American International Chemical
17 Strathmore Road
Natick, MA 01760
(800) 238-0001 (508) 655-5805
http://www.aicma.com

American Laboratory Supply
See American Bioanalytical

American Medical Systems
10700 Bren Road West
Minnetonka, MN 55343
(800) 328-3881 FAX: (612) 930-6654
(612) 933-4666
http://www.visitams.com

American Qualex
920-A Calle Negocio
San Clemente, CA 92673
(949) 492-8298 FAX: (949) 492-6790
http://www.americanqualex.com

American Radiolabeled Chemicals
11624 Bowling Green
St. Louis, MO 63146
(800) 331-6661 FAX: (800) 999-9925
(314) 991-4545 FAX: (314) 991-4692
http://www.arc-inc.com

American Scientific Products
See VWR Scientific Products

American Society for
Histocompatibility and
Immunogenetics
P.O. Box 15804
Lenexa, KS 66285
(913) 541-0009 FAX: (913) 541-0156
http://www.swmed.edu/home_pages/ASHI
/ashi.htm

American Type Culture Collection
(ATCC)
10801 University Boulevard
Manassas, VA 20110
(800) 638-6597 FAX: (703) 365-2750
(703) 365-2700
http://www.atcc.org

Amersham
See Amersham Pharmacia Biotech

Amersham International
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HP7 9NA, UK
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FAX: (44) 1494-544350
http://www.apbiotech.com

Amersham Medi-Physics
Also see Nycomed Amersham
3350 North Ridge Avenue
Arlington Heights, IL 60004
(800) 292-8514 FAX: (800) 807-2382
http://www.nycomed-amersham.com

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Piscataway, NJ 08855
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(732) 457-8000
http://www.apbiotech.com

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1 Amgen Center Drive
Thousand Oaks, CA 91320
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(805) 447-5725
http://www.amgen.com

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Scientific Systems Division
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(978) 777-3622
http://www.amicon.com

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Oakland Center
Columbia, MD 21045
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(410) 997-0100
http://www.amika.com

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See BPAmoco

AMPI
See Pacer Scientific

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Richmond, Victoria 3121, Australia
613-9208-4000
FAX: 613-9208-4350
http://www.amrad.com.au

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30175 Solon Industrial Parkway
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Rouses Point, NY 12979
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(518) 462-1066
http://www.anachemia.com

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4015 Fabian Way
Palo Alto, CA 94303
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(650) 494-3894
http://www.ana-gen.com

Analox Instruments USA
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(978) 582-9368 FAX: (978) 582-9588
http://www.analox.com

Analytical Biological Services
Cornell Business Park 701-4
Wilmington, DE 19801
(800) 391-2391 FAX: (302) 654-8046
(302) 654-4492
http://www.ABSbioreagents.com

Analytical Genetics Testing Center
7808 Cherry Creek S. Drive, Suite 201
Denver, CO 80231
(800) 204-4721 FAX: (303) 750-2171
(303) 750-2023
http://www.geneticid.com

AnaSpec
2149 O'Toole Avenue, Suite F
San Jose, CA 95131
(800) 452-5530 FAX: (408) 452-5059
(408) 452-5055
http://www.anaspec.com

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2647 Grand Avenue
P.O. Box 814
Bellmore, NY 11710
(800) 645-6379 FAX: (516) 781-4937
(516) 781-0755
http://www.ancare.com

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243 Third Street North
P.O. Box 87
Bayport, MN 55033
(800) 374-9523 FAX: (651) 439-1940
(651) 439-0835
http://www.ancell.com

Anderson Instruments
500 Technology Court
Smyrna, GA 30082
(800) 241-6898 FAX: (770) 319-5306
(770) 319-9999
http://www.graseby.com

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Gartenstrasse 100
Postfach 260
D-78732 Tuttlingen, Germany
(49) 7461 705 0
FAX: (49) 7461 705-122
http://www.hettich-centrifugen.de

Anesthetic Vaporizer Services
10185 Main Street
Clarence, NY 14031
(719) 759-8490
http://www.avapor.com

Animal Identification and
Marking Systems (AIMS)
13 Winchester Avenue
Budd Lake, NJ 07828
(908) 684-9105 FAX: (908) 684-9106
http://www.animalid.com

Annovis
34 Mount Pleasant Drive
Aston, PA 19014
(800) EASY-DNA FAX: (610) 361-8255
(610) 361-9224
http://www.annovis.com

Apotex
150 Signet Drive
Weston, Ontario
M9L 1T9 Canada
(416) 749-9300 FAX: (416) 749-2646
http://www.apotex.com

Apple Scientific
11711 Chillicothe Road, Unit 2
P.O. Box 778
Chesterland, OH 44026
(440) 729-3056 FAX: (440) 729-0928
http://www.applesci.com

Applied Biosystems
See PE Biosystems

Applied Imaging
2380 Walsh Avenue, Bldg. B
Santa Clara, CA 95051
(800) 634-3622 FAX: (408) 562-0264
(408) 562-0250
http://www.aicorp.com

Applied Photophysics
203-205 Kingston Road
Leatherhead, Surrey, KT22 7PB
UK
(44) 1372-386537

Applied Precision
1040 12th Avenue Northwest
Issaquah, Washington 98027
(425) 557-1000
FAX: (425) 557-1055
http://www.api.com/index.html

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Parc d'Innovation
Rue Geiler de Kaysersberg, BP 72
67402 Illkirch Cedex, France
(33) 88 67 22 67
FAX: (33) 88 67 19 45
http://www.oncor.com/prod-app.htm

Applikon
1165 Chess Drive, Suite G
Foster City, CA 94404
(650) 578-1396 FAX: (650) 578-8836
http://www.applikon.com

Appropriate Technical Resources
9157 Whiskey Bottom Road
Laurel, MD 20723
(800) 827-5931 FAX: (410) 792-2837
http://www.atrbiotech.com

APV Gaulin
100 S. CP Avenue
Lake Mills, WI 53551
(888) 278-4321 FAX: (888) 278-5329
http://www.apv.com

Aqualon
See Hercules Aqualon

Aquarium Systems
8141 Tyler Boulevard
Mentor, OH 44060
(800) 822-1100 FAX: (440) 255-8994
(440) 255-1997
http://www.aquariumsystems.com

Aquebogue Machine and Repair Shop
Box 2055
Main Road
Aquebogue, NY 11931
(631) 722-3635 FAX: (631) 722-3106

Archer Daniels Midland
4666 Faries Parkway
Decatur, IL 62525
(217) 424-5200
http://www.admworld.com

Archimica Florida
P.O. Box 1466
Gainesville, FL 32602
(800) 331-6313 FAX: (352) 371-6246
(352) 376-8246
http://www.archimica.com

Arcor Electronics
1845 Oak Street #15
Northfield, IL 60093
(847) 501-4848

Arcturus Engineering
400 Logue Avenue
Mountain View, CA 94043
(888) 446 7911 FAX: (650) 962 3039
(650) 962 3020
http://www.arctur.com

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887 Industrial Road, Suite G
San Carlos, CA 94070
(650) 998-1350 FAX: (650) 598-1359
http://www.argotech.com

Ariad Pharmaceuticals
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Cambridge, MA 02139
(617) 494-0400 FAX: (617) 494-8144
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See Rhone-Poulenc Rorer

Aronex Pharmaceuticals
8707 Technology Forest Place
The Woodlands, TX 77381
(281) 367-1666 FAX: (281) 367-1676
http://www.aronex.com

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73 Pond Street
Waltham, MA 02254
(617) 893-6800
http://www.artisanind.com

ASI Instruments
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Warren, MI 48089
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(810) 756-1222
http://www.asi-instruments.com

Aspen Research Laboratories
1700 Buerkle Road
White Bear Lake, MN 55140
(651) 264-6000 FAX: (651) 264-6270
http://www.aspenresearch.com

Associates of Cape Cod
704 Main Street
Falmouth, MA 02540
(800) LAL-TEST FAX: (508) 540-8680
(508) 540-3444
http://www.acciusa.com

Astra Pharmaceuticals
See AstraZeneca

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1800 Concord Pike
Wilmington, DE 19850
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http://www.astrazeneca.com

AT Biochem
30 Spring Mill Drive
Malvern, PA 19355
(610) 889-9300 FAX: (610) 889-9304

ATC Diagnostics
See Vysis

ATCC
See American Type Culture Collection

Athens Research and Technology
P.O. Box 5494
Athens, GA 30604
(706) 546-0207 FAX: (706) 546-7395

Atlanta Biologicals
1425-400 Oakbrook Drive
Norcross, GA 30093
(800) 780-7788 or (770) 446-1404
FAX: (800) 780-7374 or (770) 446-1404
http://www.atlantabio.com

Atomergic Chemical
71 Carolyn Boulevard
Farmingdale, NY 11735
(631) 694-9000 FAX: (631) 694-9177
http://www.atomergic.com

Atomic Energy of Canada
2251 Speakman Drive
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http://www.aecl.ca

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http://www.atrbiotech.com

Aurora Biosciences
11010 Torreyana Road
San Diego, CA 92121
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http://www.aurorabio.com

Automatic Switch Company
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Florham Park, NJ 07932
(800) 937-2726 FAX: (973) 966-2628
(973) 966-2000
http://www.asco.com

Avanti Polar Lipids
700 Industrial Park Drive
Alabaster, AL 35007
(800) 227-0651 FAX: (800) 229-1004
(205) 663-2494 FAX: (205) 663-0756
http://www.avantilipids.com

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http://www.aventis.com

Aventis Pasteur
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http://www.aventispasteur.com/usa

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150 North Orange Grove Boulevard
Pasadena, CA 91103
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http://www.averydennison.com

Avestin
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(613) 736-0019
http://www.avestin.com

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http://www.axon.com

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http://www.azon.com

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1223 South 47th Street
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(510) 412-8930
http://www.babco.com

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625 Alpha Drive
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http://www.bacharach-inc.com

Bachem Bioscience
3700 Horizon Drive
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(610) 239-0300
http://www.bachem.com

Bachem California
3132 Kashiwa Street
P.O. Box 3426
Torrance, CA 90510
(800) 422-2436 FAX: (310) 530-1571
(310) 539-4171
http://www.bachem.com

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18866 Allendale Avenue
Saratoga, CA 95070
(408) 972-8779 FAX: (408) 741-0944

Baker Chemical
See J.T. Baker

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Fishers, IN 46038
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http://www.bangslabs.com

Bard Parker
See Becton Dickinson

Barnstead/Thermolyne
P.O. Box 797
2555 Kerper Boulevard
Dubuque, IA 52004
(800) 446-6060 FAX: (319) 589-0516
http://www.barnstead.com

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BAS
See Bioanalytical Systems

BASF
Specialty Products
3000 Continental Drive North
Mt. Olive, NJ 07828
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http://www.basf.com

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Copiague, NY 11726
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http://www.wabaum.com

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One Bausch & Lomb Place
Rochester, NY 14604
(800) 344-8815 FAX: (716) 338-6007
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http://www.bausch.com

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1627 Lake Cook Road
Deerfield, IL 60015
(800) 766-1077 FAX: (800) 395-3291
(847) 940-6599 FAX: (847) 940-5766
http://www.powerfulmedicine.com

Baxter Healthcare
One Baxter Parkway
Deerfield, IL 60015
(800) 777-2298 FAX: (847) 948-3948
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http://www.baxter.com

Baxter Scientific Products
See VWR Scientific

Bayer
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12707 Shawnee Mission Pkwy.
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http://www.bayerus.com

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P.O. Box 2009
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(219) 256-3390
http://www.bayer.com

Bayer Diagnostics
511 Benedict Avenue
Tarrytown, NY 10591
(800) 255-3232 FAX: (914) 524-2132
(914) 631-8000
http://www.bayerdiag.com

Bayer Plc
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http://www.bdfacs.com

BD Labware
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http://www.bd.com/labware

BD PharMingen
10975 Torreyana Road
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BD Transduction Laboratories
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Palo Alto, CA 94304
(800) 742-2345 FAX: (415) 859-1550
(415) 857-1150
http://www.beckman-coulter.com

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2350 Qume Drive
San Jose, CA 95131
(800) 223-8226 FAX: (408) 954-2007
(408) 432-9475
http://www.bdfacs.com

Becton Dickinson Labware
1 Becton Drive
Franklin Lakes, NJ 07417
(888) 237-2762 FAX: (800) 847-2220
(201) 847-4222
http://www.bdfacs.com

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2 Bridgewater Lane
Lincoln Park, NJ 07035
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Care Diagnostics
7 Loveton Circle
Sparks, MD 21152
(800) 675-0908 FAX: (410) 316-4723
(410) 316-4000
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Behringwerke Diagnostika
Hoechster Strasse 70
P-65835 Liederback, Germany
(49) 69-30511 FAX: (49) 69-303-834

Bellco Glass
340 Edrudo Road
Vineland, NJ 08360
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http://www.bellcoglass.com

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(516) 273-0005
http://www.bernsco.com

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2 Ferndale Road
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FAX: (44) 1612 313656

Bethesda Research Laboratories (BRL)
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Biacore
200 Centennial Avenue, Suite 100
Piscataway, NJ 08854
(800) 242-2599 FAX: (732) 885-5669
(732) 885-5618
http://www.biacore.com

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St. Julian's
Sevenoaks, Kent TN15 0RX, UK
(44) 1732 450002
FAX: (44) 1732 450003
http://www.bilaney.com

Binding Site
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San Diego, CA 92121
(800) 633-4484 FAX: (619) 453-9189
(619) 453-9177
http://www.bindingsite.co.uk

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(800) 845-4246 FAX: (765) 497-1102
(765) 463-4527
http://www.bioanalytical.com

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Rancho Dominguez, CA 90220
(800) 222-8382 FAX: (310) 637-3927
(310) 537-3300
http://www.biocell.com

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E3B 1P6 Canada
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(506) 453-4812
http://131.202.97.21

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(914) 454-6610 FAX: (914) 454-6077
http://www.biodesignofny.com

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Marina Del Rey, CA 90292
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http://www.biodiscovery.com

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(41) 55-256-8-111
FAX: (41) 55-256-8-256

Biofluids
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Rockville, MD 20850
(800) 972-5200 FAX: (301) 424-3619
(301) 424-4140
http://www.biosource.com

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Randallstown, MD 21133
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http://www.biofx.com

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Reno, NV 89502
(888) 257-5155 FAX: (775) 828-7676
(775) 828-0202
http://www.bioline.com

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A.Z. de Font-Ratel
38640 CLAIX, France
(33) 76-98-68-31
FAX: (33) 76-98-69-09

Biological Detection Systems
See Cellomics or Amersham

Biomeda
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P.O. Box 8045
Foster City, CA 94404
(800) 341-8787 FAX: (650) 341-2299
(650) 341-8787
http://www.biomeda.com

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1 Silas Road
Seaford, DE 19973
(800) 526-2637 FAX: (302) 628-4110
(302) 628-4100
http://www.bmds.com

Biomedical Engineering
P.O. Box 980694
Virginia Commonwealth University
Richmond, VA 23298
(804) 828-9829 FAX: (804) 828-1008

Biomedical Research Instruments
12264 Wilkins Avenue
Rockville, MD 20852
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(301) 881-7911
http://www.biomedinstr.com

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Santa Monica, CA 90406
(800) 269-1158 FAX: (800) 269-1158
(323) 938-7515

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Princeton, NJ 08543
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Plymouth Meeting, PA 19462
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(610) 941-0430
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Saranac Lake, NY 12983
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http://www.bionique.com

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Santa Barbara, CA 93117
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http://www.biopac.com

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(724) 282-7145
http://www.bioptechs.com

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Hercules, CA 94547
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(510) 741-1000 FAX: (510) 741-5800
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Hemel Hempstead, Herts HP2 7TD, UK
http://www.bio-rad.com

BioRobotics
3-4 Bennell Court
Comberton, Cambridge CB3 7DS, UK
(44) 1223-264345
FAX: (44) 1223-263933
http://www.biorobotics.co.uk

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(800) GENOME1 FAX: (415) 883-8488
(415) 883-8400
http://www.biosearchtech.com

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111 Locke Drive
Marlborough, MA 01752
(800) 752-5277 FAX: (508) 357-7595
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http://www.biosepra.com

Bio-Serv
1 8th Street, Suite 1
Frenchtown, NJ 08825
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http://www.bio-serv.com

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Montreal, Quebec
H3J 1R4 Canada
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Ferguson, MO 63135
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(805) 987-0086
http://www.biosource.com

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Rockville, MD 20850
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Kimpton, Hertfordshire SG4 8PU, UK
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FAX: (44) 1438 833040
http://www.biotinst.demon.co.uk

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Australia
61-7-3370-6396
FAX: 61-7-3370-6370
http://www.avianbiotech.com

Biotech Source
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(207) 892-3266 FAX: (207) 892-6774

Bio-Tek Instruments
Highland Industrial Park
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Winooski, VT 05404
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http://www.biotek.com

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Houston, TX 77033
(800) 535-6286 FAX: (713) 643-3143
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http://www.biotecx.com

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(800) 638-8174 FAX: (301) 845-8338
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Palm Springs, CA 92262
(800) 328-4139 FAX: (760) 778-7274
(760) 778-7200
http://www.birdprod.com/bird

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2403 Yale Way
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(800) USA-MICE FAX: (510) 490-3036

BLS Ltd.
Zselyi Aladar u. 31
1165 Budapest, Hungary
(36) 1-407-2602 FAX: (36) 1-407-2896
http://www.bls-ltd.com

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San Jose, CA 95134
(408) 474-0988 FAX: (408) 474-0989
http://www.blueskyresearch.com

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New York, NY 10018
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Mundelein, IL 60060
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http://www.bpamoco.com

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http://www.brainresearchlab.com

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http://www.plasticsnet.com/branson

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999 Postal Road
Allentown, PA 18103
(800) 258-9000 FAX: (610) 266-9319
(610) 266-6262
http://www.bbraunbiotech.com

B. Braun Biotech International
Schwarzenberg Weg 73-79
P.O. Box 1120
D-34209 Melsungen, Germany
(49) 5661-71-3400
FAX: (49) 5661-71-3702
http://www.bbraunbiotech.com

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2525 McGaw Avenue
Irvine, CA 92614
(800) BBRAUN-2 (800) 624-2963
http://www.bbraunusa.com

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Thorncliffe Park
Sheffield S35 2PW, UK
(44) 114-225-9000
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http://www.bbmuk.demon.co.uk

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Princeton, NJ 08543
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http://www.bms.com

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Irvine, CA 92618
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(949) 829-5555
http://www.broadleyjames.com

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Norcross, GA 30071
(800) 332-2040 FAX: (770) 847-8440
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http://www.bkhome.com

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Madison, WI 53711
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(608) 276-3000
http://www.bruker-axs.com

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Billerica, MA 01821
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(858) 597-6006
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http://www.burnsvet.com

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http://www.wabutler.com

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TW11 8LG, UK
(44)(0)20-8977-0750
FAX: (44)(0)28-8943-2624
http://www.butterworth-labs.co.uk

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95 West Wood Road #2
Sharon, CT 06069
(860) 364-5558 FAX: (860) 364-5116
http://www.buxco.com

C/D/N Isotopes
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Pointe-Claire, Quebec
H9R 1H1 Canada
(800) 697-6254 FAX: (514) 697-6148

C.M.A./Microdialysis AB
73 Princeton Street
North Chelmsford, MA 01863
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(978) 251-1940
http://www.microdialysis.com

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(800) 854-3417 FAX: (800) 776-0999
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http://www.calbiochem.com

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Grover Beach, CA 93433
(805) 489-5144 FAX: (805) 489-5352
http://www.calfinewire.com

Calorimetry Sciences
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Spanish Fork, UT 84660
(801) 794-2600 FAX: (801) 794-2700
http://www.calscorp.com

Caltag Laboratories
1849 Bayshore Highway, Suite 200
Burlingame, CA 94010
(800) 874-4007 FAX: (650) 652-9030
(650) 652-0468
http://www.caltag.com

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Science Park, Milton Road
Cambridge CB4 0FE, UK
44 (0) 1223-420-186
FAX: 44 (0) 1223-420-488
http://www.ced.co.uk

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50 Frontage Road
Andover, MA 01810
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http://www.isotope.com

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Cambridge, MA 02138
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http://www.camtech.com

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Nuffield Road
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http://www.camlab.co.uk/home.htm

Campden Instruments
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Cappel Laboratories
See Organon Teknika Cappel

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76185 Karlsrube
Germany
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FAX: (49) 72-156-06264
http://www.carl-roth.de

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(914) 747-1800
http://www.zeiss.com

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20148 Milano
Lombardia, Italy
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Charlotte, NC 28273
(704) 588-6101 FAX: (704) 588-6115
http://www.cfcsite.com

Cartesian Technologies
17851 Skypark Circle, Suite C
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(800) 935-8007
http://cartesiantech.com

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(800) 364-9897 FAX: (734) 971-3640
(734) 971-3335
http://www.caymanchem.com

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(800) 234-1757 FAX: (603) 742-2455
http://www.cbsci.com

CBS Scientific
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Del Mar, CA 92014
(800) 243-4959 FAX: (858) 755-0733
(858) 755-4959
http://www.cbssci.com

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http://www.cellmark-labs.com

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http://www.celltech.co.uk

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(800) 726-3331

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Atlanta, GA 30333
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(404) 639-3311
http://www.cdc.gov

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53940 Le Genest Saint Isle
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Cetus
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http://www.chemgenes.com

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(800) 843-1794 FAX: (856) 696-9102
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http://www.chemglass.com

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http://www.chemservice.com

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Lenexa, KS 66215
(913) 541-0525 FAX: (913) 888-3582
http://www.tech.epcorp.com/ChemSyn/chemsyn.htm

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http://www.cherwell.com

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Malvern, PA 19355
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http://www.chromtech.com

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(802) 257-1800
http://www.chroma.com

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FAX: (33) 01-69-0988091
http://www.chromatographie.com

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(46) 31-706-20-70
FAX: (46) 31-706-20-80
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Ciba Specialty Chemicals
540 White Plains Road
Tarrytown, NY 10591
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(914) 785-2000
http://www.cibasc.com

Ciba Vision
Division of Novartis AG
11460 Johns Creek Parkway
Duluth, GA 30097
(770) 476-3937
http://www.cvworld.com

Cidex
Advanced Sterilization Products
33 Technology Drive
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(800) 595-0200 (949) 581-5799
http://www.cidex.com/ASPnew.htm

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5645 Montgomery Road
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(513) 841-0900
http://www.mrcgene.com

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1006 AD Amsterdam, The Netherlands
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FAX: (31) 20-512-3332

Cleveland Scientific
P.O. Box 300
Bath, OH 44210
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http://www.clontech.com

Closure Medical Corporation
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Raleigh, NC 27616
(919) 876-7800 FAX: (919) 790-1041
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CMA Microdialysis AB
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(800) 440-4980 FAX: (978) 251-1950
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http://www.microdialysis.com

Cocalico Biologicals
449 Stevens Road
P.O. Box 265
Reamstown, PA 17567
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5100 Patrick Henry Drive
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http://www.cohr.com

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San Diego, CA 92186
(858) 277-6700 FAX: (858) 277-0221
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http://www.cplscientific.co.uk

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(814) 443-4881
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West Gillibrands, Lancashire
WN8 9SP, UK
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FAX: (44) 1695-555518
http://www.diachem.co.uk

Diagen
Max-Volmer Strasse 4
D-40724 Hilden, Germany
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FAX: (49) 2103-892-222

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(408) 737-0700 FAX: (408) 730-9403
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Vista, CA 92083
(800) 697-1111 FAX: (760) 599-9930
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Boston, MA 02132
(617) 965-8557 FAX: (617) 323-5641
(800) 796-9199
http://www.divbio.com

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P.O. Box 121585
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(615) 298-3524
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Madison, WI 53715
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http://www.dnastar.com

DNAVIEW
Attn: Charles Brenner
http://www.wco.com
~cbrenner/dnaview.htm

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Midland, MI 48674
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http://www.dow.com

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Allesley, Coventry CV5 9RG, UK
(44) 1676 528 000
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31-0-23-5319093
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Beaufort, NC 28516-9721
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http://www.dynal.net

Dynal AS
Ullernchausen 52,
0379 Oslo, Norway
47-22-06-10-00 FAX: 47-22-50-70-15
http://www.dynal.no

Dynalab
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Rochester, NY 14692
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EC Apparatus
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Hospital for Sick Children
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Piscataway, NJ 08855
(800) 227-4750 FAX: (877) 295-8102
http://www.apbiotech.com

Hoffman-LaRoche
340 Kingsland Street
Nutley, NJ 07110
(800) 526-0189 FAX: (973) 235-9605
(973) 235-5000
http://www.rocheUSA.com

Holborn Surgical and Medical
Instruments
Westwood Industrial Estate
Ramsgate Road
Margate, Kent CT9 4JZ UK
(44) 1843 296666
FAX: (44) 1843 295446

Honeywell
101 Columbia Road
Morristown, NJ 07962
(973) 455-2000 FAX: (973) 455-4807
http://www.honeywell.com

Honeywell Specialty Films
P.O. Box 1039
101 Columbia Road
Morristown, NJ 07962
(800) 934-5679 FAX: (973) 455-6045
http://www.honeywell-specialtyfilms.com

Hood Thermo-Pad Canada
Comp. 20, Site 61A, RR2
Summerland, British Columbia
V0H 1Z0 Canada
(800) 665-9555 FAX: (250) 494-5003
(250) 494-5002
http://www.thermopad.com

Horiba Instruments
17671 Armstrong Avenue
Irvine, CA 92714
(949) 250-4811 FAX: (949) 250-0924
http://www.horiba.com

Hoskins Manufacturing
10776 Hall Road
P.O. Box 218
Hamburg, MI 48139
(810) 231-1900 FAX: (810) 231-4311
http://www.hoskinsmfgco.com

Hosokawa Micron Powder Systems
10 Chatham Road
Summit, NJ 07901
(800) 526-4491 FAX: (908) 273-7432
(908) 273-6360
http://www.hosokawamicron.com

HT Biotechnology
Unit 4
61 Ditton Walk
Cambridge CB5 8QD, UK
(44) 1223-412583

Hugo Sachs Electronik
Postfach 138
7806 March-Hugstetten, Germany
D-79229(49) 7665-92000
FAX: (49) 7665-920090

Human Biologics International
7150 East Camelback Road, Suite 245
Scottsdale, AZ 85251
(480) 990-2005 FAX: (480)-990-2155
http://www.humanbiological.com

Human Genetic Mutant Cell
Repository
See Coriell Institute for Medical Research

HVS Image
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Hampton, Middlesex TW12 2YD, UK
FAX: (44) 208 783 1223
In the US: (800) 225-9261
FAX: (888) 483-8033
http://www.hvsimage.com

Hybaid
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Teddington, Middlesex TW11 8LL, UK
(44) 0 1784 42500
FAX: (44) 0 1784 248085
http://www.hybaid.co.uk

Hybaid Instruments
8 East Forge Parkway
Franklin, MA 02028
(888)4-HYBAID FAX: (508) 541-3041
(508) 541-6918
http://www.hybaid.com

Hybridon
155 Fortune Boulevard
Milford, MA 01757
(508) 482-7500 FAX: (508) 482-7510
http://www.hybridon.com

HyClone Laboratories
1725 South HyClone Road
Logan, UT 84321
(800) HYCLONE FAX: (800) 533-9450
(801) 753-4584 FAX: (801) 750-0809
http://www.hyclone.com

Hyseq
670 Almanor Avenue
Sunnyvale, CA 94086
(408) 524-8100 FAX: (408) 524-8141
http://www.hyseq.com

IBA GmbH
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St. Louis, MO 63104
(877) 422-4624 FAX: (888) 531-6813
http://www.iba-go.com

IBF Biotechnics
See Sepracor

IBI (International Biotechnologies)
See Eastman Kodak
For technical service (800) 243-2555
(203) 786-5600

ICN Biochemicals
See ICN Biomedicals

ICN Biomedicals
3300 Hyland Avenue
Costa Mesa, CA 92626
(800) 854-0530 FAX: (800) 334-6999
(714) 545-0100 FAX: (714) 641-7275
http://www.icnbiomed.com

ICN Flow and Pharmaceuticals
See ICN Biomedicals

ICN Immunobiochemicals
See ICN Biomedicals

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ICONIX
100 King Street West, Suite 3825
Toronto, Ontario
M5X 1E3 Canada
(416) 410-2411 FAX: (416) 368-3089
http://www.iconix.com

ICRT (Imperial Cancer Research
Technology)
Sardinia House
Sardinia Street
London WC2A 3NL, UK
(44) 1712-421136
FAX: (44) 1718-314991

Idea Scientific Company
P.O. Box 13210
Minneapolis, MN 55414
(800) 433-2535 FAX: (612) 331-4217
http://www.ideascientific.com

IEC
See International Equipment Co.

IITC
23924 Victory Boulevard
Woodland Hills, CA 91367
(888) 414-4482 (818) 710-1556
FAX: (818) 992-5185
http://www.iitcinc.com

IKA Works
2635 N. Chase Parkway, SE
Wilmington, NC 28405
(910) 452-7059 FAX: (910) 452-7693
http://www.ika.net

Ikegami Electronics
37 Brook Avenue
Maywood, NJ 07607
(201) 368-9171 FAX: (201) 569-1626

Ikemoto Scientific Technology
25-11 Hongo
3-chome, Bunkyo-ku
Tokyo 101-0025, Japan
(81) 3-3811-4181
FAX: (81) 3-3811-1960

Imagenetics
See ATC Diagnostics

Imaging Research
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500 Glenridge Avenue
St. Catharines, Ontario
L2S 3A1 Canada
(905) 688-2040 FAX: (905) 685-5861
http://www.imaging.brocku.ca

Imclone Systems
180 Varick Street
New York, NY 10014
(212) 645-1405 FAX: (212) 645-2054
http://www.imclone.com

IMCO Corporation LTD., AB
P.O. Box 21195
SE-100 31
Stockholm, Sweden
46-8-33-53-09 FAX: 46-8-728-47-76
http://www.imcocorp.se

Imgenex Corporation
11175 Flintkote Avenue
Suite E
San Diego, CA 92121
(888) 723-4363 FAX: (858) 642-0937
(858) 642.0978
http://www.imgenex.com

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(34) 1-535-3960 FAX: (34) 1-535-2780

Immunex
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Seattle, WA 98101
(206) 587-0430 FAX: (206) 587-0606
http://www.immunex.com

Immunocorp
1582 W. Deere Avenue
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Irvine, CA 92606
(800) 446-3063
http://www.immunocorp.com

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13276 Marseilles Cedex 9
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(33) 491-17-27-00
FAX: (33) 491-41-43-58
http://www.immunotech.fr

Imperial Chemical Industries
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FAX: (44)171-834-2042
http://www.ici.com

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Incstar
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http://www.incyte.com

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Palo Alto, CA 94304
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(650) 855-0555
http://www.incyte.com

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Baltimore, MD 21214

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(888) 463-6346 FAX: (908) 359-1179
(908) 359-6778
http://www.indofinechemical.com

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Bronx, NY 10462
(718) 931-8000 FAX: (718) 863-1138
http://www.industrialacoustics.com

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Glenlyon Business Park
Burnaby, British Columbia
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http://www.inexpharm.com

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Wilmington, MA 01887
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http://www.mt.com

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http://www.innogenetics.com

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1725 Gillespie Way
El Cajon, CA 92020
(619) 596-8600 FAX: (619) 596-8700
http://www.imspure.com

Innovative Research
3025 Harbor Lane N, Suite 300
Plymouth, MN 55447
(612) 519-0105 FAX: (612) 519-0239
http://www.inres.com

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Sarasota, FL 34236
(800) 421-8171 FAX: (800) 643-4345
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http://www.innovrsrch.com

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(301) 670-2850
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http://www.inovision.com

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(610) 941-0132
http://www.instechlabs.com

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(781) 575-5000
http://www.instron.com

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00031 Instrumentarium
Helsinki, Finland
(10) 394-5566
http://www.instrumentarium.fi

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FAX: (33)1 6909-9319
http://www.isainc.com

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Port Washington, NY 11050
(516) 883-1300 FAX: (516) 883-1558
http://www.instrutech.com

Integrated DNA Technologies
1710 Commercial Park
Coralville, IA 52241
(800) 328-2661 FAX: (319) 626-8444
http://www.idtdna.com

Integrated Genetics
See Genzyme Genetics

Integrated Scientific Imaging Systems
3463 State Street, Suite 431
Santa Barbara, CA 93105
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http://www.imagingsystems.com

Integrated Separation Systems (ISS)
See OWL Separation Systems

IntelliGenetics
See Oxford Molecular Group

Interactiva BioTechnologie
Sedanstrasse 10
D-89077 Ulm, Germany
(49) 731-93579-290
FAX: (49) 731-93579-291
http://www.interactiva.de

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03103 France
(33) 04-70-03-83-55
FAX: (33) 04-70-03-93-60

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Falcover Road
Haverhill, Suffolk CB9 7XU, UK
(44) 1440 703460
FAX: (44) 1440 704397
http://www.interfocus.ltd.uk

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2 Manhattanville Road
Purchase, NY 10577
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(914) 694-1700 FAX: (914) 694-1429
http://www.intergenco.com

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Kaysville, UT 84037
(800) 999-2901 FAX: (800) 574-7892
(801) 547-5047 FAX: (801) 547-5051
http://www.bioexpress.com

International Biotechnologies (IBI)
See Eastman Kodak

International Equipment Co. (IEC)
See Thermoquest

International Institute for the
Advancement of Medicine
1232 Mid-Valley Drive
Jessup, PA 18434
(800) 486-IIAM FAX: (570) 343-6993
(570) 496-3400
http://www.iiam.org

International Light
17 Graf Road
Newburyport, MA 01950
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International Market Supply (I.M.S.)
Dane Mill
Broadhurst Lane
Congleton, Cheshire CW12 1LA, UK
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FAX: (44) 1260 276007

International Marketing Services
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6301 Ivy Lane, Suite 408
Greenbelt, MD 20770
(800) 373-0096 FAX: (301) 345-0631
(301) 345-2866
http://www.imvlimited.com

International Products
201 Connecticut Drive
Burlington, NJ 08016
(609) 386-8770 FAX: (609) 386-8438
http://www.mkt@ipcol.com

Intracel Corporation
Bartels Division
2005 Sammamish Road, Suite 107
Issaquah, WA 98027
(800) 542-2281 FAX: (425) 557-1894
(425) 392-2992
http://www.intracel.com

Invitrogen
1600 Faraday Avenue
Carlsbad, CA 92008
(800) 955-6288 FAX: (760) 603-7201
(760) 603-7200
http://www.invitrogen.com

In Vivo Metric
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Healdsburg, CA 95448
(707) 433-4819 FAX: (707) 433-2407

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San Diego, CA 92121
(858) 546-1300 FAX: (858) 546-3083
http://www.irori.com

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2511 Daimler Street
Santa Ana, CA 92705
(800) 577-6097 FAX: (949) 261-6522
(949) 261-7800
http://www.irvinesci.com

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Kaysville, UT 84037
(800) 999-2901 FAX: (800) 574-7892
(801) 547-5047
http://www.bioexpress.com

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4700 Superior
Lincoln, NE 68505
(800) 228-4373 FAX: (402) 464-0318
(402) 464-0231
http://www.isco.com

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Carlsbad Research Center
2292 Faraday Avenue
Carlsbad, CA 92008
(760) 931-9200
http://www.isip.com

Isolabs
See Wallac

ISS
See Integrated Separation Systems

J & W Scientific
See Agilent Technologies

J.A. Webster
86 Leominster Road
Sterling , MA 01564
(800) 225-7911 FAX: (978) 422-8959
http://www.jawebster.com

J.T. Baker
See Mallinckrodt Baker
222 Red School Lane
Phillipsburg, NJ 08865
(800) JTBAKER FAX: (908) 859-6974
http://www.jtbaker.com

Jackson ImmunoResearch
Laboratories
P.O. Box 9
872 W. Baltimore Pike
West Grove, PA 19390
(800) 367-5296 FAX: (610) 869-0171
(610) 869-4024
http://www.jacksonimmuno.com

The Jackson Laboratory
600 Maine Street
Bar Harbor, ME 04059
(800) 422-6423 FAX: (207) 288-5079
(207) 288-6000
http://www.jax.org

Jaece Industries
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North Tonawanda, NY 14120
(716) 694-2811 FAX: (716) 694-2811
http://www.jaece.com

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1125 Trenton-Harbourton Road
Titusville, NJ 09560
(609) 730-2577 FAX: (609) 730-2116
http://us.janssen.com

Jasco
8649 Commerce Drive
Easton, MD 21601
(800) 333-5272 FAX: (410) 822-7526
(410) 822-1220
http://www.jascoinc.com

Jena Bioscience
Loebstedter Str. 78
07749 Jena, Germany
(49) 3641-464920
FAX: (49) 3641-464991
http://www.jenabioscience.com

Jencons Scientific
800 Bursca Drive, Suite 801
Bridgeville, PA 15017
(800) 846-9959 FAX: (412) 257-8809
(412) 257-8861
http://www.jencons.co.uk

JEOL Instruments
11 Dearborn Road
Peabody, MA 01960
(978) 535-5900 FAX: (978) 536-2205
http://www.jeol.com/index.html

Jewett
750 Grant Street
Buffalo, NY 14213
(800) 879-7767 FAX: (716) 881-6092
(716) 881-0030
http://www.JewettInc.com

John's Scientific
See VWR Scientific

John Weiss and Sons
95 Alston Drive
Bradwell Abbey
Milton Keynes, Buckinghamshire
MK1 4HF UK
(44) 1908-318017
FAX: (44) 1908-318708

Johnson & Johnson Medical
2500 Arbrook Boulevard East
Arlington, TX 76004
(800) 423-4018
http://www.jnjmedical.com

Johnston Matthey Chemicals
Orchard Road
Royston, Hertfordshire SG8 5HE, UK
(44) 1763-253000
FAX: (44) 1763-253466
http://www.chemicals.matthey.com

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683 E. Center Street, Unit H
Grayslake, IL 60030
(847) 548-2330 FAX: (847) 548-2984
http://www.jolley.com

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See Shelton Scientific

Jorgensen Laboratories
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Loveland, CO 80538
(800) 525-5614 FAX: (970) 663-5042
(970) 669-2500
http://www.jorvet.com

JRH Biosciences and JR Scientific
13804 W. 107th Street
Lenexa, KS 66215
(800) 231-3735 FAX: (913) 469-5584
(913) 469-5580

Jule Bio Technologies
25 Science Park, #14, Suite 695
New Haven, CT 06511
(800) 648-1772 FAX: (203) 786-5489
(203) 786-5490
http://hometown.aol.com/precastgel/index.htm

K.R. Anderson
2800 Bowers Avenue
Santa Clara, CA 95051
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(408) 727-2800
http://www.kranderson.com

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1 Commerce Boulevard
P.O. Box 352440
Palm Coast, FL 32135
(904) 445-2000 FAX: (904) 446-2244
http://www.kanthal.com

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(800) KAPAK-57 FAX: (612) 541-0735
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http://www.kapak.com

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Rhön, Germany
(49) 9779-8080 FAX: (49) 9779-80888

Karl Storz
Köningin-Elisabeth Str. 60
D-14059 Berlin, Germany
(49) 30-30 69 09-0
FAX: (49) 30-30 19 452
http://www.karlstorz.de

KaVo EWL
P.O. Box 1320
D-88293 Leutkirch im Allgäu, Germany
(49) 7561-86-0 FAX: (49) 7561-86-371
http://www.kavo.com/english/startseite.htm

Keithley Instruments
28775 Aurora Road
Cleveland, OH 44139
(800) 552-1115 FAX: (440) 248-6168
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http://www.keithley.com

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2100 Maury Street, Box 70
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(515) 266-2111 FAX: (515) 266-8354
http://www.kemin.com

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Mansfield, MA 02048
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http://www.kendallhq.com

Kendro Laboratory Products
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Newtown, CT 06470
(800) 522-SPIN FAX: (203) 270-2166
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http://www.kendro.com

Kendro Laboratory Products
P.O. Box 1220
Am Kalkberg
D-3360 Osterod, Germany
(55) 22-316-213
FAX: (55) 22-316-202
http://www.heraeus-instruments.de

Kent Laboratories
23404 NE 8th Street
Redmond, WA 98053
(425) 868-6200 FAX: (425) 868-6335
http://www.kentlabs.com

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457 Bantam Road, #16
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http://www.kentscientific.com

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Penn Eagle Industrial Park
320 Rolling Ridge Drive
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http://www.keystonescientific.com

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1022 Spruce Street
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(888) 546-2531 FAX: (856) 794-9762
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http://www.kimble-kontes.com

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http://www.kinematica.ch

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LaMarque, TX 77568
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http://www.kin-tek.com

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Kontes Glass
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41-1-733-5733 FAX: 41-1-733-5734

David Kopf Instruments
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Lab-Line Instruments
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http://www.labrepco.com

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P.O. Box 1368
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Labnet International
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http://www.nationallabnet.com

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National Institute of Allergy and
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29 Jefry Lane
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Labsystems
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Lafayette Instrument
3700 Sagamore Parkway North
P.O. Box 5729
Lafayette, IN 47903
(800) 428-7545 FAX: (765) 423-4111
(765) 423-1505
http://www.lafayetteinstrument.com

Lambert Instruments
Turfweg 4
9313 TH Leutingewolde
The Netherlands
(31) 50-5018461 FAX: (31) 50-5010034
http://www.lambert-instruments.com

Lancaster Synthesis
P.O. Box 1000
Windham, NH 03087
(800) 238-2324 FAX: (603) 889-3326
(603) 889-3306
http://www.lancastersynthesis-us.com

Lancer
140 State Road 419
Winter Springs, FL 32708
(800) 332-1855 FAX: (407) 327-1229
(407) 327-8488
http://www.lancer.com

LaVision GmbH
Gerhard-Gerdes-Str. 3
D-37079
Goettingen, Germany
(49) 551-50549-0
FAX: (49) 551-50549-11
http://www.lavision.de

Lawshe
See Advanced Process Supply

Laxotan
20, rue Leon Blum
26000 Valence, France
(33) 4-75-41-91-91
FAX: (33) 4-75-41-91-99
http://www.latoxan.com

LC Laboratories
165 New Boston Street
Woburn, MA 01801
(781) 937-0777 FAX: (781) 938-5420
http://www.lclaboratories.com

LC Packings
80 Carolina Street
San Francisco, CA 94103
(415) 552-1855 FAX: (415) 552-1859
http://www.lcpackings.com

LC Services
See LC Laboratories

LECO
3000 Lakeview Avenue
St. Joseph, MI 49085
(800) 292-6141 FAX: (616) 982-8977
(616) 985-5496
http://www.leco.com

Lederle Laboratories
See Wyeth-Ayerst

Lee Biomolecular Research
Laboratories
11211 Sorrento Valley Road, Suite M
San Diego, CA 92121
(858) 452-7700

The Lee Company
2 Pettipaug Road
P.O. Box 424
Westbrook, CT 06498
(800) LEE-PLUG FAX: (860) 399-7058
(860) 399-6281
http://www.theleeco.com

Lee Laboratories
1475 Athens Highway
Grayson, GA 30017
(800) 732-9150 FAX: (770) 979-9570
(770) 972-4450
http://www.leelabs.com

Leica
111 Deer Lake Road
Deerfield, IL 60015
(800) 248-0123 FAX: (847) 405-0147
(847) 405-0123
http://www.leica.com

Leica Microsystems
Imneuenheimer Feld 518
D-69120
Heidelberg, Germany
(49) 6221-41480
FAX: (49) 6221-414833
http://www.leica-microsystems.com

Leinco Technologies
359 Consort Drive
St. Louis, MO 63011
(314) 230-9477 FAX: (314) 527-5545
http://www.leinco.com

Leitz U.S.A.
See Leica

LenderKing Metal Products
8370 Jumpers Hole Road
Millersville, MD 21108
(410) 544-8795 FAX: (410) 544-5069
http://www.lenderking.com

Letica Scientific Instruments
Panlab s.i., c/Loreto 50
08029 Barcelona, Spain
(34) 93-419-0709
FAX: (34) 93-419-7145
http://www.panlab-sl.com

Leybold-Heraeus Trivac DZA
5700 Mellon Road
Export, PA 15632
(412) 327-5700

LI-COR
Biotechnology Division
4308 Progressive Avenue
Lincoln, NE 68504
(800) 645-4267 FAX: (402) 467-0819
(402) 467-0700
http://www.licor.com

Life Science Laboratories
See Adaptive Biosystems

Life Science Resources
Two Corporate Center Drive
Melville, NY 11747
(800) 747-9530 FAX: (516) 844-5114
(516) 844-5085
http://www.astrocam.com

Life Sciences
2900 72nd Street North
St. Petersburg, FL 33710
(800) 237-4323 FAX: (727) 347-2957
(727) 345-9371
http://www.lifesci.com

Life Technologies
9800 Medical Center Drive
P.O. Box 6482
Rockville, MD 20849
(800) 828-6686 FAX: (800) 331-2286
http://www.lifetech.com

Lifecodes
550 West Avenue
Stamford, CT 06902
(800) 543-3263 FAX: (203) 328-9599
(203) 328-9500
http://www.lifecodes.com

Lightnin
135 Mt. Read Boulevard
Rochester, NY 14611
(888) MIX-BEST FAX: (716) 527-1742
(716) 436-5550
http://www.lightnin-mixers.com

Linear Drives
Luckyn Lane, Pipps Hill
Basildon, Essex SS14 3BW, UK
(44) 1268-287070
FAX: (44) 1268-293344
http://www.lineardrives.com

Linscott's Directory
4877 Grange Road
Santa Rosa, CA 95404
(707) 544-9555 FAX: (415) 389-6025
http://www.linscottsdirectory.co.uk

Linton Instrumentation
Unit 11, Forge Business Center
Upper Rose Lane
Palgrave, Diss, Norfolk IP22 1AP, UK
(44) 1-379-651-344
FAX: (44) 1-379-650-970
http://www.lintoninst.co.uk

List Biological Laboratories
501-B Vandell Way
Campbell, CA 95008
(800) 726-3213 FAX: (408) 866-6364
(408) 866-6363
http://www.listlabs.com

LKB Instruments
See Amersham Pharmacia Biotech

Lloyd Laboratories
604 West Thomas Avenue
Shenandoah, IA 51601
(800) 831-0004 FAX: (712) 246-5245
(712) 246-4000
http://www.lloydinc.com

Loctite
1001 Trout Brook Crossing
Rocky Hill, CT 06067
(860) 571-5100 FAX: (860)571-5465
http://www.loctite.com

Lofstrand Labs
7961 Cessna Avenue
Gaithersburg, MD 20879
(800) 541-0362 FAX: (301) 948-9214
(301) 330-0111
http://www.lofstrand.com

Lomir Biochemical
99 East Main Street
Malone, NY 12953
(877) 425-3604 FAX: (518) 483-8195
(518) 483-7697
http://www.lomir.com

LSL Biolafitte
10 rue de Temara
7810C St.-Germain-en-Laye, France
(33) 1-3061-5260
FAX: (33) 1-3061-5234

Ludl Electronic Products
171 Brady Avenue
Hawthorne, NY 10532
(888) 769-6111 FAX: (914) 769-4759
(914) 769-6111
http://www.ludl.com

Lumigen
24485 W. Ten Mile Road
Southfield, MI 48034
(248) 351-5600 FAX: (248) 351-0518
http://www.lumigen.com

Luminex
12212 Technology Boulevard
Austin, TX 78727
(888) 219-8020 FAX: (512) 258-4173
(512) 219-8020
http://www.luminexcorp.com

LYNX Therapeutics
25861 Industrial Boulevard
Hayward, CA 94545
(510) 670-9300 FAX: (510) 670-9302
http://www.lynxgen.com

Lyphomed
3 Parkway North
Deerfield, IL 60015
(847) 317-8100 FAX: (847) 317-8600

M.E.D. Associates
See Med Associates

Macherey-Nagel
6 South Third Street, #402
Easton, PA 18042
(610) 559-9848 FAX: (610) 559-9878
http://www.macherey-nagel.com

Macherey-Nagel
Valencienner Strasse 11
P.O. Box 101352
D-52313 Dueren, Germany
(49) 2421-969141
FAX: (49) 2421-969199
http://www.macherey-nagel.ch

Mac-Mod Analytical
127 Commons Court
Chadds Ford, PA 19317
800-441-7508 FAX: (610) 358-5993
(610) 358-9696
http://www.mac-mod.com

Mallinckrodt Baker
222 Red School Lane
Phillipsburg, NJ 08865
(800) 582-2537 FAX: (908) 859-6974
(908) 859-2151
http://www.mallbaker.com

Mallinckrodt Chemicals
16305 Swingley Ridge Drive
Chesterfield, MD 63017
(314) 530-2172 FAX: (314) 530-2563
http://www.mallchem.com

Malven Instruments
Enigma Business Park
Grovewood Road
Malven, Worchestershire
WR 141 XZ, United Kingdom

Marinus
1500 Pier C Street
Long Beach, CA 90813
(562) 435-6522 FAX: (562) 495-3120

Markson Science
c/o Whatman Labs Sales
P.O. Box 1359
Hillsboro, OR 97123
(800) 942-8626 FAX: (503) 640-9716
(503) 648-0762

Marsh Biomedical Products
565 Blossom Road
Rochester, NY 14610
(800) 445-2812 FAX: (716) 654-4810
(716) 654-4800
http://www.biomar.com

Marshall Farms USA
5800 Lake Bluff Road
North Rose, NY 14516
(315) 587-2295
e-mail: info@marfarms.com

Martek
6480 Dobbin Road
Columbia, MD 21045
(410) 740-0081 FAX: (410) 740-2985
http://www.martekbio.com

Martin Supply
Distributor of Gerber Scientific
2740 Loch Raven Road
Baltimore, MD 21218
(800) 282-5440 FAX: (410) 366-0134
(410) 366-1696

Mast Immunosystems
630 Clyde Court
Mountain View, CA 94043
(800) 233-MAST FAX: (650) 969-2745
(650) 961-5501
http://www.mastallergy.com

Matheson Gas Products
P.O. Box 624
959 Route 46 East
Parsippany, NJ 07054
(800) 416-2505 FAX: (973) 257-9393
(973) 257-1100
http://www.mathesongas.com

Mathsoft
1700 Westlake Avenue N., Suite 500
Seattle, WA 98109
(800) 569-0123 FAX: (206) 283-8691
(206) 283-8802
http://www.mathsoft.com

Matreya
500 Tressler Street
Pleasant Gap, PA 16823
(814) 359-5060 FAX: (814) 359-5062
http://www.matreya.com

Matrigel
See Becton Dickinson Labware

Matrix Technologies
22 Friars Drive
Hudson, NH 03051
(800) 345-0206 FAX: (603) 595-0106
(603) 595-0505
http://www.matrixtechcorp.com

MatTek Corp.
200 Homer Avenue
Ashland, Massachusetts 01721
(508) 881-6771 FAX: (508) 879-1532
http://www.mattek.com

Maxim Medical
89 Oxford Road
Oxford OX2 9PD
United Kingdom
44 (0)1865-865943
FAX: 44 (0)1865-865291
http://www.maximmed.com

Mayo Clinic
Section on Engineering
Project #ALA-1, 1982
200 1st Street SW
Rochester, MN 55905
(507) 284-2511 FAX: (507) 284-5988

McGaw
See B. Braun-McGaw

McMaster-Carr
600 County Line Road
Elmhurst, IL 60126
(630) 833-0300 FAX: (630) 834-9427
http://www.mcmaster.com

McNeil Pharmaceutical
See Ortho McNeil Pharmaceutical

MCNC
3021 Cornwallis Road
P.O. Box 12889
Research Triangle Park, NC 27709
(919) 248-1800 FAX: (919) 248-1455
http://www.mcnc.org

MD Industries
5 Revere Drive, Suite 415
Northbrook, IL 60062
(800) 421-8370 FAX: (847) 498-2627
(708) 339-6000
http://www.mdindustries.com

MDS Nordion
447 March Road
P.O. Box 13500
Kanata, Ontario
K2K 1X8 Canada
(800) 465-3666 FAX: (613) 592-6937
(613) 592-2790
http://www.mds.nordion.com

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71 Four Valley Drive
Concord, Ontario
Canada L4K 4V8
(905) 660-9005 FAX: (905) 660-2600
http://www.sciex.com

Mead Johnson
See Bristol-Meyers Squibb

Med Associates
P.O. Box 319
St. Albans, VT 05478
(802) 527-2343 FAX: (802) 527-5095
http://www.med-associates.com

Medecell
239 Liverpool Road
London N1 1LX, UK
(44) 20-7607-2295
FAX: (44) 20-7700-4156
http://www.medicell.co.uk

Media Cybernetics
8484 Georgia Avenue, Suite 200
Silver Spring, MD 20910
(301) 495-3305 FAX: (301) 495-5964
http://www.mediacy.com

Mediatech
13884 Park Center Road
Herndon, VA 20171
(800) cellgro
(703) 471-5955
http://www.cellgro.com

Medical Systems
See Harvard Apparatus

Medifor
647 Washington Street
Port Townsend, WA 98368
(800) 366-3710 FAX: (360) 385-4402
(360) 385-0722
http://www.medifor.com

MedImmune
35 W. Watkins Mill Road
Gaithersburg, MD 20878
(301) 417-0770 FAX: (301) 527-4207
http://www.medimmune.com

MedProbe AS
P.O. Box 2640
St. Hanshaugen
N-0131 Oslo, Norway
(47) 222 00137 FAX: (47) 222 00189
http://www.medprobe.com

Megazyme
Bray Business Park
Bray, County Wicklow
Ireland
(353) 1-286-1220
FAX: (353) 1-286-1264
http://www.megazyme.com

Melles Griot
4601 Nautilus Court South
Boulder, CO 80301
(800) 326-4363 FAX: (303) 581-0960
(303) 581-0337
http://www.mellesgriot.com

Menzel-Glaser
Postfach 3157
D-38021 Braunschweig, Germany
(49) 531 590080
FAX: (49) 531 509799

E. Merck
Frankfurterstrasse 250
D-64293 Darmstadt 1, Germany
(49) 6151-720

Merck
See EM Science

Merck & Company
Merck National Service Center
P.O. Box 4
West Point, PA 19486
(800) NSC-MERCK
(215) 652-5000
http://www.merck.com

Merck Research Laboratories
See Merck & Company

Merck Sharpe Human Health Division
300 Franklin Square Drive
Somerset, NJ 08873
(800) 637-2579 FAX: (732) 805-3960
(732) 805-0300

Merial Limited
115 Transtech Drive
Athens, GA 30601
(800) MERIAL-1 FAX: (706) 548-0608
(706) 548-9292
http://www.merial.com

Meridian Instruments
P.O. Box 1204
Kent, WA 98035
(253) 854-9914 FAX: (253) 854-9902
http://www.minstrument.com

Meta Systems Group
32 Hammond Road
Belmont, MA 02178
(617) 489-9950 FAX: (617) 489-9952

Metachem Technologies
3547 Voyager Street, Bldg. 102
Torrance, CA 90503
(310) 793-2300 FAX: (310) 793-2304
http://www.metachem.com

Metallhantering
Box 47172
100-74 Stockholm, Sweden
(46) 8-726-9696

MethylGene
7220 Frederick-Banting, Suite 200
Montreal, Quebec
H4S 2A1 Canada
http://www.methylgene.com

Metro Scientific
475 Main Street, Suite 2A
Farmingdale, NY 11735
(800) 788-6247 FAX: (516) 293-8549
(516) 293-9656

Metrowerks
980 Metric Boulevard
Austin, TX 78758
(800) 377-5416
(512) 997-4700
http://www.metrowerks.com

Mettler Instruments
Mettler-Toledo
1900 Polaris Parkway
Columbus, OH 43240
(800) METTLER FAX: (614) 438-4900
http://www.mt.com

Miami Serpentarium Labs
34879 Washington Loop Road
Punta Gorda, FL 33982
(800) 248-5050 FAX: (813) 639-1811
(813) 639-8888
http://www.miamiserpentarium.com

Michrom BioResources
1945 Industrial Drive
Auburn, CA 95603
(530) 888-6498 FAX: (530) 888-8295
http://www.michrom.com

Mickle Laboratory Engineering
Gomshall, Surrey, UK
(44) 1483-202178

Micra Scientific
A division of Eichrom Industries
8205 S. Cass Ave, Suite 111
Darien, IL 60561
(800) 283-4752 FAX: (630) 963-1928
(630) 963-0320
http://www.micrasci.com

MicroBrightField
74 Hegman Avenue
Colchester, VT 05446
(802) 655-9360 FAX: (802) 655-5245
http://www.microbrightfield.com

Micro Essential Laboratory
4224 Avenue H
Brooklyn, NY 11210
(718) 338-3618 FAX: (718) 692-4491

Micro Filtration Systems
7-3-Chome, Honcho
Nihonbashi, Tokyo, Japan
(81) 3-270-3141

Micro-Metrics
P.O. Box 13804
Atlanta, GA 30324
(770) 986-6015 FAX: (770) 986-9510
http://www.micro-metrics.com

Micro-Tech Scientific
140 South Wolfe Road
Sunnyvale, CA 94086
(408) 730-8324 FAX: (408) 730-3566
http://www.microlc.com

Microbix Biosystems
341 Bering Avenue
Toronto, Ontario
M8Z 3A8 Canada
1-800-794-6694 FAX: 416-234-1626
1-416-234-1624
http://www.microbix.com

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22 Industrial Drive East
Northampton, MA 01060
(800) 633-3115 FAX: (413) 586-0149
(413) 586-7720
http://www.microcalorimetry.com

Microfluidics
30 Ossipee Road
P.O. Box 9101
Newton, MA 02164
(800) 370-5452 FAX: (617) 965-1213
(617) 969-5452
http://www.microfluidicscorp.com

Microgon
See Spectrum Laboratories

Microlase Optical Systems
West of Scotland Science Park
Kelvin Campus, Maryhill Road
Glasgow G20 0SP, UK
(44) 141-948-1000
FAX: (44) 141-946-6311
http://www.microlase.co.uk

Micron Instruments
4509 Runway Street
Simi Valley, CA 93063
(800) 638-3770 FAX: (805) 522-4982
(805) 552-4676
http://www.microninstruments.com

Micron Separations
See MSI

Micro Photonics
4949 Liberty Lane, Suite 170
P.O. Box 3129
Allentown, PA 18106
(610) 366-7103 FAX: (610) 366-7105
http://www.microphotonics.com

MicroTech
1420 Conchester Highway
Boothwyn, PA 19061
(610) 459-3514

Midland Certified Reagent Company
3112-A West Cuthbert Avenue
Midland, TX 79701
(800) 247-8766 FAX: (800) 359-5789
(915) 694-7950 FAX: (915) 694-2387
http://www.mcrc.com

Midwest Scientific
280 Vance Road
Valley Park, MO 63088
(800) 227-9997 FAX: (636) 225-9998
(636) 225-9997
http://www.midsci.com

Miles
See Bayer

Miles Laboratories
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Miles Scientific
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Millar Instruments
P.O. Box 230227
6001-A Gulf Freeway
Houston, TX 77023
(713) 923-9171 FAX: (713) 923-7757
http://www.millarinstruments.com

MilliGen/Biosearch
See Millipore

Millipore
80 Ashbury Road
P.O. Box 9125
Bedford, MA 01730
(800) 645-5476 FAX: (781) 533-3110
(781) 533-6000
http://www.millipore.com

Miltenyi Biotec
251 Auburn Ravine Road, Suite 208
Auburn, CA 95603
(800) 367-6227 FAX: (530) 888-8925
(530) 888-8871
http://www.miltenyibiotec.com

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6 Ohio Drive
Lake Success, NY 11042
(800) 645-8000 FAX: (516) 775-7185
(516) 349-0001

Milton Roy
See Spectronic Instruments

Mini-Instruments
15 Burnham Business Park
Springfield Road
Burnham-on-Crouch, Essex CM0 8TE, UK
(44) 1621-783282
FAX: (44) 1621-783132
http://www.mini-instruments.co.uk

Mini Mitter
P.O. Box 3386
Sunriver, OR 97707
(800) 685-2999 FAX: (541) 593-5604
(541) 593-8639
http://www.minimitter.com

Mirus Corporation
505 S. Rosa Road
Suite 104
Madison, WI 53719
(608) 441-2852 FAX: (608) 441-2849
http://www.genetransfer.com

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1938 New Highway
Farmingdale, NY 11735
(800) 645-9846 FAX: (516) 694-9412
http://www.misonix.com

Mitutoyo (MTI)
See Dolla Eastern

MJ Research
Waltham, MA 02451
(800) PELTIER FAX: (617) 923-8080
(617) 923-8000
http://www.mjr.com

Modular Instruments
228 West Gay Street
Westchester, PA 19380
(610) 738-1420 FAX: (610) 738-1421
http://www.mi2.com

Molecular Biology Insights
8685 US Highway 24
Cascade, CO 80809-1333
(800) 747-4362 FAX: (719) 684-7989
(719) 684-7988
http://www.oligo.net

Molecular Biosystems
10030 Barnes Canyon Road
San Diego, CA 92121
(858) 452-0681 FAX: (858) 452-6187
http://www.mobi.com

Molecular Devices
1312 Crossman Avenue
Sunnyvale, CA 94089
(800) 635-5577 FAX: (408) 747-3602
(408) 747-1700
http://www.moldev.com

Molecular Designs
1400 Catalina Street
San Leandro, CA 94577
(510) 895-1313 FAX: (510) 614-3608

Molecular Dynamics
928 East Arques Avenue
Sunnyvale, CA 94086
(800) 333-5703 FAX: (408) 773-1493
(408) 773-1222
http://www.apbiotech.com

Molecular Probes
4849 Pitchford Avenue
Eugene, OR 97402
(800) 438-2209 FAX: (800) 438-0228
(541) 465-8300 FAX: (541) 344-6504
http://www.probes.com

Molecular Research Center
5645 Montgomery Road
Cincinnati, OH 45212
(800) 462-9868 FAX: (513) 841-0080
(513) 841-0900
http://www.mrcgene.com

Molecular Simulations
9685 Scranton Road
San Diego, CA 92121
(800) 756-4674 FAX: (858) 458-0136
(858) 458-9990
http://www.msi.com

Monoject Disposable Syringes & Needles/Syrvet
16200 Walnut Street
Waukee, IA 50263
(800) 727-5203 FAX: (515) 987-5553
(515) 987-5554
http://www.syrvet.com

Monsanto Chemical
800 North Lindbergh Boulevard
St. Louis, MO 63167
(314) 694-1000 FAX: (314) 694-7625
http://www.monsanto.com

Moravek Biochemicals
577 Mercury Lane
Brea, CA 92821
(800) 447-0100 FAX: (714) 990-1824
(714) 990-2018
http://www.moravek.com

Moss
P.O. Box 189
Pasadena, MD 21122
(800) 932-6677 FAX: (410) 768-3971
(410) 768-3442
http://www.mosssubstrates.com

Motion Analysis
3617 Westwind Boulevard
Santa Rosa, CA 95403
(707) 579-6500 FAX: (707) 526-0629
http://www.motionanalysis.com

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Farmington Industrial Park
84 Spring Lane
Farmington, CT 06032
(860) 747-6333 FAX: (860) 747-6739
http://www.mottcorp.com

MSI (Micron Separations)
See Osmonics

Multi Channel Systems
Markwiesenstrasse 55
72770 Reutlingen, Germany
(49) 7121-503010
FAX: (49) 7121-503011
http://www.multichannelsystems.com

Multiple Peptide Systems
3550 General Atomics Court
San Diego, CA 92121
(800) 338-4965 FAX: (800) 654-5592
(858) 455-3710 FAX: (858) 455-3713
http://www.mps-sd.com

Murex Diagnostics
3075 Northwoods Circle
Norcross, GA 30071
(707) 662-0660 FAX: (770) 447-4989

MWG-Biotech
Anzinger Str. 7
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http://www.mwg_biotech.com

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http://www.myriadindustries.com

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(516) 794-8000
http://www.narishige.co.jp

National Bag Company
2233 Old Mill Road
Hudson, OH 44236
(800) 247-6000 FAX: (330) 425-9800
(330) 425-2600
http://www.nationalbag.com

National Band and Tag
Department X 35, Box 72430
Newport, KY 41032
(606) 261-2035 FAX: (800) 261-8247
https://www.nationalband.com

National Biosciences
See Molecular Biology Insights

National Diagnostics
305 Patton Drive
Atlanta, GA 30336
(800) 526-3867 FAX: (404) 699-2077
(404) 699-2121
http://www.nationaldiagnostics.com

National Institute of Standards and Technology
100 Bureau Drive
Gaithersburg, MD 20899
(301) 975-NIST FAX: (301) 926-1630
http://www.nist.gov

National Instruments
11500 North Mopac Expressway
Austin, TX 78759
(512) 794-0100 FAX: (512) 683-8411
http://www.ni.com

National Labnet
See Labnet International

National Scientific Instruments
975 Progress Circle
Lawrenceville, GA 300243
(800) 332-3331 FAX: (404) 339-7173
http://www.nationalscientific.com

National Scientific Supply
1111 Francisco Bouldvard East
San Rafael, CA 94901
(800) 525-1779 FAX: (415) 459-2954
(415) 459-6070
http://www.nat-sci.com

Naz-Dar-KC Chicago
Nazdar
1087 N. North Branch Street
Chicago, IL 60622
(800) 736-7636 FAX: (312) 943-8215
(312) 943-8338
http://www.nazdar.com

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1918 Avenue A
Denison, TX 75021
(903) 465-2694 FAX: (903) 463-5905
http://www.nblabslarry.com

NEB
See New England Biolabs

NEN Life Science Products
549 Albany Street
Boston, MA 02118
(800) 551-2121 FAX: (617) 451-8185
(617) 350-9075
http://www.nen.com

NEN Research Products, Dupont (UK)
Diagnostics and Biotechnology Systems
Wedgewood Way
Stevenage, Hertfordshire SG1 4QN, UK
44-1438-734831
44-1438-734000
FAX: 44-1438-734836
http://www.dupont.com

Neogen
628 Winchester Road
Lexington, KY 40505
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(606) 254-1221
http://www.neogen.com

Neosystems
380, 11012 Macleod Trail South
Calgary, Alberta
T2J 6A5 Canada
(403) 225-9022 FAX: (403) 225-9025
http://www.neosystems.com

Neuralynx
2434 North Pantano Road
Tucson, AZ 85715
(520) 722-8144 FAX: (520) 722-8163
http://www.neuralynx.com

Neuro Probe
16008 Industrial Drive
Gaithersburg, MD 20877
(301) 417-0014 FAX: (301) 977-5711
http://www.neuroprobe.com

Neurocrine Biosciences
10555 Science Center Drive
San Diego, CA 92121
(619) 658-7600 FAX: (619) 658-7602
http://www.neurocrine.com

Nevtek
HCR03, Box 99
Burnsville, VA 24487
(540) 925-2322 FAX: (540) 925-2323
http://www.nevtek.com

New Brunswick Scientific
44 Talmadge Road
Edison, NJ 08818
(800) 631-5417 FAX: (732) 287-4222
(732) 287-1200
http://www.nbsc.com

New England Biolabs (NEB)
32 Tozer Road
Beverly, MA 01915
(800) 632-5227 FAX: (800) 632-7440
http://www.neb.com

New England Nuclear (NEN)
See NEN Life Science Products

New MBR
Gubelstrasse 48
CH8050 Zurich, Switzerland
(41) 1-313-0703

Newark Electronics
4801 N. Ravenswood Avenue
Chicago, IL 60640
(800) 4-NEWARK FAX: (773) 907-5339
(773) 784-5100
http://www.newark.com

Newell Rubbermaid
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Freeport, IL 61032
(815) 235-4171 FAX: (815) 233-8060
http://www.newellco.com

Newport Biosystems
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Red Bluff, CA 96080
(530) 529-2448 FAX: (530) 529-2648

Newport
1791 Deere Avenue
Irvine, CA 92606
(800) 222-6440 FAX: (949) 253-1800
(949) 253-1462
http://www.newport.com

Nexin Research B.V.
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4740 AA Hoeven, The Netherlands
(31) 165-503172
FAX: (31) 165-502291

NIAID
See Bio-Tech Research Laboratories

Nichiryo
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Flanders, NJ 07836
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(973) 927-4001
http://www.nichiryo.com

Nichols Institute Diagnostics
33051 Calle Aviador
San Juan Capistrano, CA 92675
(800) 286-4NID FAX: (949) 240-5273
(949) 728-4610
http://www.nicholsdiag.com

Nichols Scientific Instruments
3334 Brown Station Road
Columbia, MO 65202
(573) 474-5522 FAX: (603) 215-7274
http://home.beseen.com
technology/nsi_technology

Nicolet Biomedical Instruments
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Madison, WI 53711
(800) 356-0007 FAX: (608) 441-2002
(608) 273-5000
http://nicoletbiomedical.com

N.I.G.M.S. (National Institute of
General Medical Sciences)
See Coriell Institute for Medical Research

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Science and Technologies Group
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Melville, NY 11747
(516) 547-8500 FAX: (516) 547-4045
http://www.nikonusa.com

Nippon Gene
1-29, Ton-ya-machi
Toyama 930, Japan
(81) 764-51-6548
FAX: (81) 764-51-6547

Noldus Information Technology
751 Miller Drive
Suite E-5
Leesburg, VA 20175
(800) 355-9541 FAX: (703) 771-0441
(703) 771-0440
http://www.noldus.com

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North American Biologicals (NABI)
16500 NW 15th Avenue
Miami, FL 33169
(800) 327-7106 (305) 625-5305
http://www.nabi.com

North American Reiss
See Reiss

Northwestern Bottle
24 Walpole Park South
Walpole, MA 02081
(508) 668-8600 FAX: (508) 668-7790

NOVA Biomedical
Nova Biomedical 200
Prospect Street Waltham, MA 02454
(800) 822-0911 FAX: (781) 894-5915
http://www.novabiomedical.com

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Madison, WI 53711
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(608) 238-6110
http://www.novagen.com

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59 Route 10
East Hanover, NJ 07936
(800)526-0175 FAX: (973) 781-6356
http://www.novartis.com

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3054 Cornwallis Road
Research Triangle Park, NC 27709
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http://www.novartis.com

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Subsidiary of Airflow Lufttechnik GmbH
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Novex/Invitrogen
1600 Faraday
Carlsbad, CA 92008
(800) 955-6288 FAX: (760) 603-7201
http://www.novex.com

Novo Nordisk Biochem
77 Perry Chapel Church Road
Franklington, NC 27525
(800) 879-6686 FAX: (919) 494-3450
(919) 494-3000
http://www.novo.dk

Novo Nordisk BioLabs
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Balliol Business Park West
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Newcastle-upon-Tyne
Tyne and Wear NE12 8EW, UK
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FAX: (44) 191-215-1152
http://www.novocastra.co.uk

Novus Biologicals
P.O. Box 802
Littleton, CO 80160
(888) 506-6887 FAX: (303) 730-1966
http://www.novus-biologicals.com/main.html

NPI Electronic
Hauptstrasse 96
D-71732 Tamm, Germany
(49) 7141-601534
FAX: (49) 7141-601266
http://www.npielectronic.com

NSG Precision Cells
195G Central Avenue
Farmingdale, NY 11735
(516) 249-7474 FAX: (516) 249-8575
http://www.nsgpci.com

Nu Chek Prep
109 West Main
P.O. Box 295
Elysian, MN 56028
(800) 521-7728 FAX: (507) 267-4790
(507) 267-4689

Nuclepore
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Montgomeryville, PA 18936
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(215) 362-2766
http://www.interactivewhiteboards.com

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c/o Accurate Chemical & Scientific
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Westbury, NY 11590
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(516) 333-2221
http://www.accuratechemical.com

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Health Care Division
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Princeton, NJ 08540
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(609) 514-6000
http://www.nycomed-amersham.com

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Ohmeda Catheter Products
See Datex-Ohmeda

Ohwa Tsusbo
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Tokyo 100, Japan
03-3591-7348 FAX: 03-3501-9001

Oligos Etc.
9775 S.W. Commerce Circle, C-6
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(800) 888-2358 FAX: (503) 6822D1635
(503) 6822D1814
http://www.oligoetc.com

Olis Instruments
130 Conway Drive
Bogart, GA 30622
(706) 353-6547 (800) 852-3504
http://www.olisweb.com

Olympus America
2 Corporate Center Drive
Melville, NY 11747
(800) 645-8160 FAX: (516) 844-5959
(516) 844-5000
http://www.olympusamerica.com

Omega Engineering
One Omega Drive
P.O. Box 4047
Stamford, CT 06907
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(203) 359-1660
http://www.omega.com

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3 Grove Street
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Brattleboro, VT 05302
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http://www.omegafilters.com

Omnetics Connector Corporation
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East Minneapolis, MN 55432
(800) 343-0025 (763) 572-0656
Fax: (763) 572-3925
http://www.omnetics.com/main.htm

Omni International
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Warrenton, VA 20187
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(540) 347-5331
http://www.omni-inc.com

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2010 Energy Drive
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http://www.omnion.com

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http://www.apoptosis.com

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Online Instruments
130 Conway Drive, Suites A & B
Bogart, GA 30622
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(706) 353-6547
http://www.olisweb.com

Operon Technologies
1000 Atlantic Avenue
Alameda, CA 94501
(800) 688-2248 FAX: (510) 865-5225
(510) 865-8644
http://www.operon.com

Optiscan
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Mount Waverly MDC, Victoria
Australia 3149
61-3-9538 3333 FAX: 61-3-9562 7742
http://www.optiscan.com.au

Optomax
9 Ash Street
P.O. Box 840
Hollis, NH 03049
(603) 465-3385 FAX: (603) 465-2291

Opto-Line Associates
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Wilmington, MA 01887
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http://www.optoline.com

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San Diego, CA 92121
(866) 672-4436 (858) 362-2030
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http://www.orbigen.com

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Lawrence, KS 66047
(800) 447-6501 FAX: (785) 749-1882
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http://www.oread.com

Organomation Associates
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Berlin, MA 01503
(888) 978-7300 FAX: (978)838-2786
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http://www.organomation.com

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West Orange, NJ 07052
(800) 241-8812 FAX: (973) 325-4589
(973) 325-4500
http://www.organon.com

Organon Teknika (Canada)
30 North Wind Place
Scarborough, Ontario
M1S 3R5 Canada
(416) 754-4344 FAX: (416) 754-4488
http://www.organonteknika.com

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Durham, NC 27712
(800) 682-2666 FAX: (800) 432-9682
(919) 620-2000 FAX: (919) 620-2107
http://www.organonteknika.com

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Stratford, CT 06615
(203) 377-8282 FAX: (203) 378-2457
http://www.oriel.com

OriGene Technologies
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Rockville, MD 20850
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http://www.origene.com

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Northhampton, MA 01060
(800) 969-7720 FAX: (413) 585-0126
http://www.originlab.com

Orion Research
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Beverly, MA 01915
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(978) 232-6000
http://www.orionres.com

Ortho Diagnostic Systems
Subsidiary of Johnson & Johnson
1001 U.S. Highway 202
P.O. Box 350
Raritan, NJ 08869
(800) 322-6374 FAX: (908) 218-8582
(908) 218-1300

Ortho McNeil Pharmaceutical
Welsh & McKean Road
Spring House, PA 19477
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(215) 628-5000
http://www.orthomcneil.com

Oryza
200 Turnpike Road, Unit 5
Chelmsford, MA 01824
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http://www.oryzalabs.com

OSI Pharmaceuticals
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Uniondale, NY 11553
(800) 662-2616 FAX: (516) 222-0114
(516) 222-0023
http://www.osip.com

Osmonics
135 Flanders Road
P.O. Box 1046
Westborough, MA 01581
(800) 444-8212 FAX: (508) 366-5840
(508) 366-8212
http://www.osmolabstore.com

Oster Professional Products
150 Cadillac Lane
McMinnville, TN 37110
(931) 668-4121 FAX: (931) 668-4125
http://www.sunbeam.com

Out Patient Services
1260 Holm Road
Petaluma, CA 94954
(800) 648-1666 FAX: (707) 762-7198
(707) 763-1581

OWL Scientific Plastics
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OWL Separation Systems
55 Heritage Avenue
Portsmouth, NH 03801
(800) 242-5560 FAX: (603) 559-9258
(603) 559-9297
http://www.owlsci.com

Oxford Biochemical Research
P.O. Box 522
Oxford, MI 48371
(800) 692-4633 FAX: (248) 852-4466
http://www.oxfordbiomed.com

Oxford GlycoSystems
See Glyco

Oxford Instruments
Old Station Way
Eynsham
Witney, Oxfordshire OX8 1TL, UK
(44) 1865-881437
FAX: (44) 1865-881944
http://www.oxinst.com

Oxford Labware
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Oxford Molecular Group
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http://www.oxmol.co.uk

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http://www.oxmol.com

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Portland, OR 97217
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http://www.oxis.com

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800 Proctor Avenue
Ogdensburg, NY 13669
(800) 567-8378 FAX: (613) 226-3728
http://www.oxoid.ca

Oxoid
Wade Road
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(44) 1256-841144
FAX: (4) 1256-814626
http://www.oxoid.ca

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Mansfield, OH 44901
(419) 589-8800 FAX: (419) 589-9919
http://www.oxyrase.com

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10 Avenue Ampère
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78180 France
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http://www.ozyme.fr

PAA Laboratories
2570 Route 724
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(610) 495-9400 FAX: (610) 495-9410
http://www.paa-labs.com

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Los Angeles, CA 90068
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http://www.pacersci.com

Pacific Bio-Marine Labs
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800 Research Parkway
Meriden, CT 06450
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(203) 238-2351
http://www.packardinst.com

Padgett Instrument
1730 Walnut Street
Kansas City, MO 64108
(816) 842-1029

Pall Filtron
50 Bearfoot Road
Northborough, MA 01532
(800) FILTRON FAX: (508) 393-1874
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Pall-Gelman
25 Harbor Park Drive
Port Washington, NY 11050
(800) 289-6255 FAX: (516) 484-2651
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http://www.pall.com

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545 Science Drive
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(800) 791-1400 FAX: (608) 233-3007
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http://www.panvera.com

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Moline, IL 61265
(800) 872-7720 FAX: (309) 762-9453
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http://www.parrinst.com

Partec
Otto Hahn Strasse 32
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PCR
See Archimica Florida

PE Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
(800) 345-5224 FAX: (650) 638-5884
(650) 638-5800
http://www.pebio.com

Pel-Freez Biologicals
219 N. Arkansas
P.O. Box 68
Rogers, AR 72757
(800) 643-3426 FAX: (501) 636-3562
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http://www.pelfreez-bio.com

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(650) 592-5392
http://www.penlabs.com

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24562 Mando Drive
Laguna Niguel, CA 92677
(800) 382-4667 FAX: (714) 643-2363
http://www.pentex.com

PeproTech
5 Crescent Avenue
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(609) 497-0253
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http://www.peptide.co.jp

Peptide Laboratory
4175 Lakeside Drive
Richmond, CA 94806
(800) 858-7322 FAX: (510) 262-9127
(510) 262-0800
http://www.peptidelab.com

Peptides International
11621 Electron Drive
Louisville, KY 40299
(800) 777-4779 FAX: (502) 267-1329
(502) 266-8787
http://www.pepnet.com

Perceptive Science Instruments
2525 South Shore Boulevard, Suite 100
League City, TX 77573
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http://www.persci.com

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4873 Princeton Drive
North Royalton, OH 44133
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http://www.perimed.se

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Norwalk, CT 06859
(800) 762-4002 FAX: (203) 762-6000
(203) 762-1000
http://www.perkin-elmer.com
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PerSeptive Bioresearch Products
See PerSeptive BioSystems

PerSeptive BioSystems
500 Old Connecticut Path
Framingham, MA 01701
(800) 899-5858 FAX: (508) 383-7885
(508) 383-7700
http://www.pbio.com

PerSeptive Diagnostic
See PE Biosystems
(800) 343-1346

Pettersson Elektronik AB
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http://www.bahnhof.se/~pettersson

Pfanstiehl Laboratories, Inc.
1219 Glen Rock Avenue
Waukegan, IL 60085
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http://www.pfanstiehl.com

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7311 Governors Way
Frederick, MD 21704
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http://www.pgcscientifics.com

Pharmacia Biotech
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See Wallac

Pharmacia LKB Biotech
See Amersham Pharmacia Biotech

Pharmacia LKB Biotechnology
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Pharmacia LKB Nuclear
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Pharmaderm Veterinary Products
60 Baylis Road
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http://www.pharmaderm.com

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Norton Performance Plastics
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PharMingen
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Phenomex
2320 W. 205th Street
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(310) 212-0555 FAX: (310) 328-7768
http://www.phenomex.com

PHLS Centre for Applied
Microbiology and Research
See European Collection of Animal
Cell Cultures (ECACC)

Phoenix Flow Systems
11575 Sorrento Valley Road, Suite 208
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http://www.phnxflow.com

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4261 Easton Road, P.O. Box 6457
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(816) 364-5777
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Photometrics
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Photon Technology International
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http://www.pti-nj.com

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Polytec PI
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Auburn, MA 01501
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Physitemp Instruments
154 Huron Avenue
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(800) 522-2469 FAX: (603) 788-3873
(603) 788-4952
http://www.pjnoyes.com

Plas-Labs
917 E. Chilson Street
Lansing, MI 48906
(800) 866-7527 FAX: (517) 372-2857
(517) 372-7177
http://www.plas-labs.com

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P.O. Box 12004
Roanoke, VA 24018
(540) 772-7950 FAX: (540) 989-7519
http://www.plastics1.com

Platt Electric Supply
2757 6th Avenue South
Seattle, WA 98134
(206) 624-4083 FAX: (206) 343-6342
http://www.platt.com

Plexon
6500 Greenville Avenue
Suite 730
Dallas,TX 75206
(214) 369-4957 FAX: (214) 369-1775
http://www.plexoninc.com

Polaroid
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Cambridge, MA 01239
(800) 225-1618 FAX: (800) 832-9003
(781) 386-2000
http://www.polaroid.com

Polyfiltronics
136 Weymouth St.
Rockland, MA 02370
(800) 434-7659 FAX: (781) 878-0822
(781) 878-1133
http://www.polyfiltronics.com

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10, rue de la Durance
B.P. 36
67023 Strasbourg Cedex 1
Strasbourg, France
33-3-8865-8020
FAX: 33-3-8865-8039

PolyLC
9151 Rumsey Road, Suite 180
Columbia, MD 21045
(410) 992-5400 FAX: (410) 730-8340

Polymer Laboratories
Amherst Research Park
160 Old Farm Road
Amherst, MA 01002
(800) 767-3963 FAX: (413) 253-2476
http://www.polymerlabs.com

Polymicro Technologies
18019 North 25th Avenue
Phoenix, AZ 85023
(602) 375-4100 FAX: (602) 375-4110
http://www.polymicro.com

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Hanaveien 4-6
4327 Sandnes, Norway
(47) 51-62-0990
FAX: (47) 51-62-51-82
http://www.polyphenols.com

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400 Valley Road
Warrington, PA 18976
(800) 523-2575 FAX: (800) 343-3291
http://www.polysciences.com

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Bayshore, NY 11706
(516) 586-0400 FAX: (516) 254-0618

Polytech Products
285 Washington Street
Somerville, MA 02143
(617) 666-5064 FAX: (617) 625-0975

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8585 Grovemont Circle
Gaithersburg, MD 20877
(301) 208-6597 FAX: (301) 208-8691
http://www.polytron.com

Popper and Sons
300 Denton Avenue
P.O. Box 128
New Hyde Park, NY 11040
(888) 717-7677 FAX: (800) 557-6773
(516) 248-0300 FAX: (516) 747-1188
http://www.popperandsons.com

Porphyrin Products
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Logan, UT 84323
(435) 753-1901 FAX: (435) 753-6731
http://www.porphyrin.com

Portex
See SIMS Portex Limited

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585 Science Drive
Madison, WI 53711
(608) 231-3150 FAX: (608) 231-6990
http://www.powderject.com

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810 Jorie Boulevard
Oak Brook, IL 60521
(800) 621-7100
http://www.praxair.com

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13880 Del Sur Street
San Fernando, CA 91340
(800) 847-0670 FAX: (818) 899-4-45
http://www.pdcorp.com

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(540) 869-9892
http://www.precisionsci.com

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See Becton Dickinson Primary Care Diagnostics

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Redwood City, CA 94063
(650) 368-0663 FAX: (650) 368-0665
http://www.primateproducts.com

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http://www.5prime.com

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PerkinElmer Instr.: Electrochemistry
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Oak Ridge, TN 37830
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(423) 481-2442
http://www.eggpar.com

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http://www.prinst.com

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Aldephia, NJ 07710
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(732) 431-3338

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Rockland, MA 02370
(781) 878-8442 FAX: (781) 878-8736
http://www.prior.com

PRO Scientific
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Monroe, CT 06468
(203) 452-9431 FAX: (203) 452-9753
http://www.proscientific.com

Professional Compounding Centers of America
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Houston, TX 77099
(800) 331-2498 FAX: (281) 933-6227
(281) 933-6948
http://www.pccarx.com

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Maass-Str. 30
69123 Heidelberg, Germany
(49) 6221-8278-0
FAX: (49) 6221-8278-23
http://www.progen.de

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54 rue Roger Salengro
94126 Fontenay Sous Bois Cedex
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http://www.prolabo.fr

Proligo
2995 Wilderness Place
Boulder, CO 80301
(888) 80-OLIGO FAX: (303) 801-1134
http://www.proligo.com

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2800 Woods Hollow Road
Madison, WI 53711
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(608) 274-4330 FAX: (608) 277-2516
http://www.promega.com

Protein Databases (PDI)
405 Oakwood Road
Huntington Station, NY 11746
(800) 777-6834 FAX: (516) 673-4502
(516) 673-3939

Protein Polymer Technologies
10655 Sorrento Valley Road
San Diego, CA 92121
(619) 558-6064 FAX: (619) 558-6477
http://www.ppti.com

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391 G Chipeta Way
Salt Lake City, UT 84108
(801) 583-9301 FAX: (801) 583-4463
http://www.proteinsolutions.com

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1933 Davis Street, Suite 207
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(510) 638-6900
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See Perceptive Science Instruments

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Chestnut Hill, MA 02167
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http://www.pharma.com

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http://www.purina-mills.com

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7581 Lindbergh Drive
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http://www.qualitybiological.com

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http://www.quantum-appligene.com

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San Diego, CA 92121
(800) 874-1517 FAX: (858) 546-8955
(858) 552-1100
http://www.quidel.com

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http://www.r-biopharm.com

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Santa Barbara, CA 93117
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http://www.rcelectronics.com

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Minneapolis, MN 55413
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(612) 379-2956
http://www.rndsystems.com

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Peacedale, RI 02880
(401) 789-5660 FAX: (401) 792-3890
http://www.septech.com

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7305 Executive Way
Frederick, MD 21704
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Radiometer America
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Westlake, OH 44145
(800) 736-0600 FAX: (440) 871-2633
(440) 871-8900
http://www.rameusa.com

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The Chemical Reference Laboratory
kandevej 21
DK-2700 Brnshj, Denmark
45-3827-3827 FAX: 45-3827-2727

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(781) 272-1233 FAX: (781) 272-2428
http://www.radionics.com

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(800) 428-l4l6 FAX: (626) 303-2998
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http://www.rainin.com

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CB5 9DA UK
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FAX: (44) 1223 811441
http://www.rankbrothers.com

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FAX: (49) 7071-763158
http://www.rapp-polymere.com

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P.O. Box 27261
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(800) 728-5702 FAX: (402) 593-0995
(402) 593-0781
http://www.ravenlabs.com

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Stamford, CT 06906
(203) 324-9914 FAX: (203) 324-5568

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See Biotech Source

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10000 Virginia Manor Road, Suite 360
Beltsville, MD 20705
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http://www.receptorbiology.com

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http://www.registech.com

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Buffalo, NY 14240
(716) 686-4500 FAX: (716) 686-4545
http://www.reichert.com

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(800) 356-2829 FAX: (804) 292-1757
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http://www.reissmfg.com

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P.O. Box 14428
Shawnee Mission, KS 66215
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(913) 888-0939 FAX: (913) 888-5884
http://www.remelinc.com

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Redfield, NY 13437
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Needham, MA 02494
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(781) 449-9560
http://www.repligen.com

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Natick, MA 01760
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(508) 651-8151 FAX: (508) 655-1359
http://www.resbio.com

Research Corporation Technologies
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Tucson, AZ 85711
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http://www.rctech.com

Research Diagnostics
Pleasant Hill Road
Flanders, NJ 07836
(800) 631-9384 FAX: (973) 584-0210
(973) 584-7093
http://www.researchd.com

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121 Jersey Avenue
New Brunswick, NJ 08901
(877) 486-2486 FAX: (732) 247-2340
(732) 247-2390
http://www.researchdiets.com

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Huntsville, AL 35801
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(256) 533-4363
http://www.resgen.com

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Cornwall TR10 9DQ, UK
(44) 1326-372-753
FAX: (44) 1326-378-783
http://www.research-instruments.com

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Cleveland, OH 44125
(800) 321-0570 FAX: (216) 883-1576
(216) 883-8025
http://www.resorg.com

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Bayonne, NJ 07002
(800) 341-2296 FAX: (201) 823-9590
(201) 823-3592
http://www.researchplus.com

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Mount Prospect, IL 60056
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(847) 635-7330
http://www.rpicorp.com

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Research Triangle Park, NC 27709
(919) 541-6000 FAX: (919) 541-6515
http://www.rti.org

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(814) 353-1300
http://www.restekcorp.com

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(707) 588-2000 FAX: (707) 588-2020
http://www.rheodyne.com

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Rhone-Poulenc
2 T W Alexander Drive
P.O. Box 12014
Research Triangle Park, NC 08512
(919) 549-2000 FAX: (919) 549-2839
http://www.Rhone-Poulenc.com
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Rhone-Poulenc Rorer
500 Arcola Road
Collegeville, PA 19426
(800) 727-6737 FAX: (610) 454-8940
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http://www.rp-rorer.com

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Centre de Recherche de Vitry-Alfortville
13 Quai Jules Guesde, BP14 94403
Vitry Sur Seine, Cedex, France
(33) 145-73-85-11
FAX: (33) 145-73-81-29
http://www.rp-rorer.com

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Hamilton, MT 59840
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(406) 363-3131
http://www.ribi.com

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(888) GO-RICCA FAX: (800) RICCA-93
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(616) 344-2400
http://www.rallansci.com

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11 177 Hamon
Montral, Quebec
H3M 3E4 Canada
(802) 863-2567 FAX: (802) 862-2909
http://www.richelieubio.com

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Wayland, MA 01778
(508) 655-7632 FAX: (508) 652-7264
http://www.richter-enterprises.com

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BioSure Division
12301 G Loma Rica Drive
Grass Valley, CA 95945
(800) 345-2267 FAX: (916) 273-5097
(916) 273-5095
http://www.biosure.com

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Sunnyvale, CA 94086
(800) 752-8585 FAX: (408) 734-0300
(408) 734-8500
http://www.robsci.com

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Rockville, MD 20850
(800) 424-2984 FAX: (301) 590-1290
(301) 590-0055

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Indianapolis, IN 46256
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(317) 845-2000
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FAX: (64) 9-634-7696
http://www.rocklabs.com

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D-64293 Darmstadt, Germany
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http://www.roperscientific.com

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12040 115th Avenue NE
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(425) 820-8900 FAX: (425) 820-5757
http://www.rii.com

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Lauterbourg
67630 France
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FAX: (33) 03-88-54-63-93

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Huntington, NY 11743
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http://www.rotronic-usa.com

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Pewaukee, WI 53072
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http://www.roundys.com

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FAX: (44) 1536 405678
http://www.rs-components.com

Rubbermaid
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831-761-1000 FAX: 831-761-1008
http://www.sageinst.com

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(508) 653-0844 FAX: 508-653-5671
http://www.sagelabs.com

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Akron, OH 44309
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http://www.nortonplastics.com

San Diego Instruments
7758 Arjons Drive
San Diego, CA 92126
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http://www.sd-inst.com

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25 Oldfield Road
Hampden, Middlesex TW12 2AJ, UK
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FAX: (44) 2089 793311
http://www.sandownsci.com

Sandoz Pharmaceuticals
See Novartis

Sanofi Recherche
Centre de Montpellier
371 Rue du Professor Blayac
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Sanofi Winthrop Pharmaceuticals
90 Park Avenue
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http://www.sanofi-synthelabo.com/us

Santa Cruz Biotechnology
2161 Delaware Avenue
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http://www.scbt.com

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http://www.scanalytics.com

Schering Laboratories
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http://www.schering-plough.com

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http://www.s-und-s.de/english-index.html

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http://www.scientificinstruments.com

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http://www.scottgas.com

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http://www.sensormedics.com

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http://www.vsensors.com

SenSym/Foxboro ICT
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(408) 954-6700
http://www.sensym.com

Separations Group
See Vydac

Sepracor
111 Locke Drive
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See Harlan Sera-Lab

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(49) 030-779-9060

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619 West Oak Street
P.O. Box 1529
Oak Harbor, WA 98277
(800) 426-0191 FAX: (800) 359-3460
(360) 679-2528 FAX: (360) 679-3830
http://www.upchurch.com

Upjohn
Pharmacia & Upjohn
http://www.pnu.com

Upstate Biotechnology (UBI)
1100 Winter Street, Suite 2300
Waltham, MA 02451
(800) 233-3991 FAX: (781) 890-7738
(781) 890-8845
http://www.upstatebiotech.com

USA/Scientific
346 SW 57th Avenue
P.O. Box 3565
Ocala, FL 34478
(800) LAB-TIPS FAX: (352) 351-2057
(3524) 237-6288
http://www.usascientific.com

USB
26111 Miles Road
P.O. Box 22400
Cleveland, OH 44122
(800) 321-9322 FAX: (800) 535-0898
FAX: (216) 464-5075
http://www.usbweb.com

USCI Bard
Bard Interventional Products
129 Concord Road
Billerica, MA 01821
(800) 225-1332 FAX: (978) 262-4805
http://www.bardinterventional.com

UVP (Ultraviolet Products)
2066 W. 11th Street
Upland, CA 91786
(800) 452-6788 FAX: (909) 946-3597
(909) 946-3197
http://www.uvp.com

V & P Scientific
9823 Pacific Heights Boulevard, Suite T
San Diego, CA 92121
(800) 455-0644 FAX: (858) 455-0703
(858) 455-0643
http://www.vp-scientific.com

Valco Instruments
P.O. Box 55603
Houston, TX 77255
(800) FOR-VICI FAX: (713) 688-8106
(713) 688-9345
http://www.vici.com

Valpey Fisher
75 South Street
Hopkin, MA 01748
(508) 435-6831 FAX: (508) 435-5289
http://www.valpeyfisher.com

Value Plastics
3325 Timberline Road
Fort Collins, CO 80525
(800) 404-LUER FAX: (970) 223-0953
(970) 223-8306
http://www.valueplastics.com

Vangard International
P.O. Box 308
3535 Rt. 66, Bldg. #4
Neptune, NJ 07754
(800) 922-0784 FAX: (732) 922-0557
(732) 922-4900
http://www.vangard1.com

Varian Analytical Instruments
2700 Mitchell Drive
Walnut Creek, CA 94598
(800) 926-3000 FAX: (925) 945-2102
(925) 939-2400
http://www.varianinc.com

Varian Associates
3050 Hansen Way
Palo Alto, CA 94304
(800) 544-4636 FAX: (650) 424-5358
(650) 493-4000
http://www.varian.com

Vector Core Laboratory/
National Gene Vector Labs
University of Michigan
3560 E MSRB II
1150 West Medical Center Drive
Ann Arbor, MI 48109
(734) 936-5843 FAX: (734) 764-3596

Vector Laboratories
30 Ingold Road
Burlingame, CA 94010
(800) 227-6666 FAX: (650) 697-0339
(650) 697-3600
http://www.vectorlabs.com

Vedco
2121 S.E. Bush Road
St. Joseph, MO 64504
(888) 708-3326 FAX: (816) 238-1837
(816) 238-8840
http://database.vedco.com

Ventana Medical Systems
3865 North Business Center Drive
Tucson, AZ 85705
(800) 227-2155 FAX: (520) 887-2558
(520) 887-2155
http://www.ventanamed.com

Verity Software House
P.O. Box 247
45A Augusta Road
Topsham, ME 04086
(207) 729-6767 FAX: (207) 729-5443
http://www.vsh.com

Vernitron
See Sensor Systems LLC

Vertex Pharmaceuticals
130 Waverly Street
Cambridge, MA 02139
(617) 577-6000 FAX: (617) 577-6680
http://www.vpharm.com

Vetamac
Route 7, Box 208
Frankfort, IN 46041
(317) 379-3621

Vet Drug
Unit 8
Lakeside Industrial Estate
Colnbrook, Slough SL3 0ED, UK

Vetus Animal Health
See Burns Veterinary Supply

Viamed
15 Station Road
Cross Hills, Keighley
W. Yorkshire BD20 7DT, UK
(44) 1-535-634-542
FAX: (44) 1-535-635-582
http://www.viamed.co.uk

Vical
9373 Town Center Drive, Suite 100
San Diego, CA 92121
(858) 646-1100 FAX: (858) 646-1150
http://www.vical.com

Victor Medical
2349 North Watney Way, Suite D
Fairfield, CA 94533
(800) 888-8908 FAX: (707) 425-6459
(707) 425-0294

Virion Systems
9610 Medical Center Drive, Suite 100
Rockville, MD 20850
(301) 309-1844 FAX: (301) 309-0471
http://www.radix.net/~virion

VirTis Company
815 Route 208
Gardiner, NY 12525
(800) 765-6198 FAX: (914) 255-5338
(914) 255-5000
http://www.virtis.com

Visible Genetics
700 Bay Street, Suite 1000
Toronto, Ontario
M5G 1Z6 Canada
(888) 463-6844 (416) 813-3272
http://www.visgen.com

Vitrocom
8 Morris Avenue
Mountain Lakes, NJ 07046
(973) 402-1443 FAX: (973) 402-1445

VTI
7650 W. 26th Avenue
Hialeah, FL 33106
(305) 828-4700 FAX: (305) 828-0299
http://www.vticorp.com

VWR Scientific Products
200 Center Square Road
Bridgeport, NJ 08014
(800) 932-5000 FAX: (609) 467-5499
(609) 467-2600
http://www.vwrsp.com

Vydac
17434 Mojave Street
P.O. Box 867
Hesperia, CA 92345
(800) 247-0924 FAX: (760) 244-1984
(760) 244-6107
http://www.vydac.com

Vysis
3100 Woodcreek Drive
Downers Grove, IL 60515
(800) 553-7042 FAX: (630) 271-7138
(630) 271-7000
http://www.vysis.com

W&H Dentalwerk Bürmoos
P.O. Box 1
A-5111 Bürmoos, Austria
(43) 6274-6236-0
FAX: (43) 6274-6236-55
http://www.wnhdent.com

Wako BioProducts
See Wako Chemicals USA

Wako Chemicals USA
1600 Bellwood Road
Richmond, VA 23237
(800) 992-9256 FAX: (804) 271-7791
(804) 271-7677
http://www.wakousa.com

Wako Pure Chemicals
1-2, Doshomachi 3-chome
Chuo-ku, Osaka 540-8605, Japan
81-6-6203-3741 FAX: 81-6-6222-1203
http://www.wako-chem.co.jp/egaiyo/index.htm

Wallac
See Perkin-Elmer

Wallac
A Division of Perkin-Elmer
3985 Eastern Road
Norton, OH 44203
(800) 321-9632 FAX: (330) 825-8520
(330) 825-4525
http://www.wallac.com

Waring Products
283 Main Street
New Hartford, CT 06057
(800) 348-7195 FAX: (860) 738-9203
(860) 379-0731
http://www.waringproducts.com

Warner Instrument
1141 Dixwell Avenue
Hamden, CT 06514
(800) 599-4203 FAX: (203) 776-1278
(203) 776-0664
http://www.warnerinstrument.com

Warner-Lambert
Parke-Davis
201 Tabor Road
Morris Plains, NJ 07950
(973) 540-2000 FAX: (973) 540-3761
http://www.warner-lambert.com

Washington University Machine Shop
615 South Taylor
St. Louis, MO 63310
(314) 362-6186 FAX: (314) 362-6184

Waters Chromatography
34 Maple Street
Milford, MA 01757
(800) 252-HPLC FAX: (508) 478-1990
(508) 478-2000
http://www.waters.com

Watlow
12001 Lackland Road
St. Louis, MO 63146
(314) 426-7431 FAX: (314) 447-8770
http://www.watlow.com

Watson-Marlow
220 Ballardvale Street
Wilmington, MA 01887
(978) 658-6168 FAX: (978) 988 0828
http://www.watson-marlow.co.uk

Waukesha Fluid Handling
611 Sugar Creek Road
Delavan, WI 53115
(800) 252-5200 FAX: (800) 252-5012
(414) 728-1900 FAX: (414) 728-4608
http://www.waukesha-cb.com

WaveMetrics
P.O. Box 2088
Lake Oswego, OR 97035
(503) 620-3001 FAX: (503) 620-6754
http://www.wavemetrics.com

Weather Measure
P.O. Box 41257
Sacramento, CA 95641
(916) 481-7565

Weber Scientific
2732 Kuser Road
Hamilton, NJ 08691
(800) FAT-TEST FAX: (609) 584-8388
(609) 584-7677
http://www.weberscientific.com

Weck, Edward & Company
1 Weck Drive
Research Triangle Park, NC 27709
(919) 544-8000

Wellcome Diagnostics
See Burroughs Wellcome

Wellington Laboratories
398 Laird Road
Guelph, Ontario
N1G 3X7 Canada
(800) 578-6985 FAX: (519) 822-2849
http://www.well-labs.com

Wesbart Engineering
Daux Road
Billingshurst, West Sussex
RH14 9EZ, UK
(44) 1-403-782738
FAX: (44) 1-403-784180
http://www.wesbart.co.uk

Whatman
9 Bridewell Place
Clifton, NJ 07014
(800) 631-7290 FAX: (973) 773-3991
(973) 773-5800
http://www.whatman.com

Wheaton Science Products
1501 North 10th Street
Millville, NJ 08332
(800) 225-1437 FAX: (800) 368-3108
(856) 825-1100 FAX: (856) 825-1368
http://www.algroupwheaton.com

Whittaker Bioproducts
See BioWhittaker

Wild Heerbrugg
Juerg Dedual Gaebrisstrasse 8 CH
9056 Gais, Switzerland
(41) 71-793-2723
FAX: (41) 71-726-5957
http://www.homepage.swissonline.net/dedual/wild_heerbrugg

Willy A. Bachofen AG Maschinenfabrik
Utengasse 15/17
CH4005 Basel, Switzerland
(41) 61-681-5151
FAX: (41) 61-681-5058
http://www.wab.ch

Winthrop
See Sterling Winthrop

Wolfram Research
100 Trade Center Drive
Champaign, IL 61820
(800) 965-3726 FAX: (217) 398-0747
(217) 398-0700
http://www.wolfram.com

World Health Organization
Microbiology and Immunology Support
20 Avenue Appia
1211 Geneva 27, Switzerland
(41-22) 791-2602
FAX: (41-22) 791-0746
http://www.who.org

World Precision Instruments
175 Sarasota Center Boulevard
International Trade Center
Sarasota, FL 34240
(941) 371-1003 FAX: (941) 377-5428
http://www.wpiinc.com

Worthington Biochemical
Halls Mill Road
Freehold, NJ 07728
(800) 445-9603 FAX: (800) 368-3108
(732) 462-3838 FAX: (732) 308-4453
http://www.worthington-biochem.com

WPI
See World Precision Instruments

Wyeth-Ayerst
2 Esterbrook Lane
Cherry Hill, NJ 08003
(800) 568-9938 FAX: (858) 424-8747
(858) 424-3700

Wyeth-Ayerst Laboratories
P.O. Box 1773
Paoli, PA 19301
(800) 666-7248 FAX: (610) 889-9669
(610) 644-8000
http://www.ahp.com

Xenotech
3800 Cambridge Street
Kansas City, KS 66103
(913) 588-7930 FAX: (913) 588-7572
http://www.xenotechllc.com

Xeragon
19300 Germantown Road
Germantown, MD 20874
(240) 686-7860 FAX: (240)686-7861
http://www.xeragon.com

Xillix Technologies
300-13775 Commerce Parkway
Richmond, British Columbia
V6V 2V4 Canada
(800) 665-2236 FAX: (604) 278-3356
(604) 278-5000
http://www.xillix.com

Xomed Surgical Products
6743 Southpoint Drive N
Jacksonville, FL 32216
(800) 874-5797 FAX: (800) 678-3995
(904) 296-9600 FAX: (904) 296-9666
http://www.xomed.com

Yakult Honsha
1-19, Higashi-Shinbashi 1-chome
Minato-ku Tokyo 105-8660, Japan
81-3-3574-8960

Yamasa Shoyu
23-8 Nihonbashi Kakigaracho
1-chome, Chuoku
Tokyo, 103 Japan
(81) 3-479 22 0095
FAX: (81) 3-479 22 3435

Yeast Genetic Stock Center
See ATCC

Yellow Spring Instruments
See YSI

YMC
YMC Karasuma-Gojo Building
284 Daigo-Cho, Karasuma Nisihiirr
Gojo-dori Shimogyo-ku
Kyoto, 600-8106, Japan
(81) 75-342-4567
FAX: (81) 75-342-4568
http://www.ymc.co.jp

YSI
1725-1700 Brannum Lane
Yellow Springs, OH 45387
(800) 765-9744 FAX: (937) 767-9353
(937) 767-7241
http://www.ysi.com

Zeneca/CRB
See AstraZeneca
(800) 327-0125 FAX: (800) 321-4745

Zivic-Miller Laboratories
178 Toll Gate Road
Zelienople, PA 16063
(800) 422-LABS FAX: (724) 452-4506
(800) MBM-RATS FAX: (724) 452-5200
http://zivicmiller.com

Zymark
Zymark Center
Hopkinton, MA 01748
(508) 435-9500 FAX: (508) 435-3439
http://www.zymark.com

Zymed Laboratories
458 Carlton Court
South San Francisco, CA 94080
(800) 874-4494 FAX: (650) 871-4499
(650) 871-4494
http://www.zymed.com

Zymo Research
625 W. Katella Avenue, Suite 30
Orange, CA 92867
(888) 882-9682 FAX: (714) 288-9643
(714) 288-9682
http://www.zymor.com

Zynaxis Cell Science
See ChiRex Cauldron