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High Pressure Fluid Technology for Green Food Processing

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High Pressure Fluid Technology for Green Food Processing

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Foreword

The book *High Pressure Fluid Technology for Green Food Processing* provides an overview on the application of Green-Chemistry principles to production processes and analytical procedures related to food materials, mainly using supercritical fluids and pressurized phases. The book provides an excellent summary on the state of art for processes and fundamentals.

Experimental and processing techniques for supercritical fluids and for high pressure processing have been investigated extensively in the last decades. The number of publications on specialized applications is enormous, as is the number of papers on phase equilibria and solubility. Attention has also been dedicated to determine the properties of substances and mixtures, to kinetic issues, and to reactive transformations. The formulation of solid products by means of supercritical fluids has led to new products in the food sector, while the modification of surfaces is still in an early development state. Attention has also been directed to the application of Chemical Engineering methods for processes with supercritical fluids and pressurized phases.

Fundamentals such as phase equilibria, mass transfer kinetics, application of various supercritical fluids, basically carbon dioxide, but also including other compounds as modifying component or alternative to CO_2, solvent cycles, energy requirement, and more have been investigated and are known in principle and applied in commercial processes for quite a number of products. It is self-evident that knowledge on these topics must be acquired for many more feed materials, pure components as well as for extract mixtures, usually in connection with actual commercial projects.

It has been realized—and the book is paying attention to that—that supercritical fluid processes for themselves may not lead to the wanted products. Furthermore, a combination of supercritical fluid processing with conventional processes is necessary in most cases for a process sequence leading to the saleable product. Consideration must be given to economic aspects, mainly seen from an engineering point of view as processing cost, but a wider view is necessary since marketing of the products is an essential feature.

The book is divided into three major areas: Fundamentals (Chaps. 1–4), advances in high pressure food processing (Chaps. 5–8), and current and future applications (Chaps. 9–14). In the first part, phase equilibria and their application to processes, determination of thermophysical properties, and mass transfer aspects are treated. The complex nature of food related materials leads to a variety of methods that need to be selectively used depending on the feed material. In this part, the connection between fundamentals and process parameters is given thorough attention.

In the second part, specialized and emerging techniques are described, such as the formulation of solids using supercritical fluids or dense gases, the transformation of compounds by enzymatically catalyzed reactions in a supercritical fluid environment, the use of Supercritical Fluid Chromatography for analytical and preparative purposes, and the applications of water in the sub- and supercritical state for food analysis, with the prospect for process applications. This part shows the variability of high pressure processing techniques and makes clear the availability of many techniques that are nowadays only scarcely used for commercial processes.

In the third part, current and future applications are addressed, mainly extractive processes, such as recovery of bioactive compounds from by-products, extraction of compounds from spices and herbs, extraction of carotenoids, and processing of lipids. In this part also process technology and economic aspects are presented, like multiple unit processes in particular for biorefineries and the economic perspectives of high pressure processing.

This book is an excellent overview on the state of the art applying supercritical fluids and pressurized phases, or more general—High Pressure Techniques—to the production and analysis of food materials. It is a must for all persons, students, engineers, scientists, and managers working in the field or interested in production methods that comply with Green-Chemistry methods.

Gerd Brunner

Preface

High pressure separation and reaction processes have opened a wide range of new alternatives for the expansion of food technology. Their most attractive advantage is the application of food substances (carbon dioxide, water, ethanol) as solvents which can process both lipophilic and hydrophilic raw matters. This is currently of particular interest, since during the first decade of the twenty-first century the food industry has clearly focused on generating products with proved health benefits. Since meeting the targets of green processing and green products is a requirement in the production of functional foods, the use of high pressure green solvents is appropriate and desirable as they guarantee safe and high quality products.

Part I of this book presents the basics of the synthesis and design of high pressure processes, which are strongly dependent on the phase equilibrium scenario, which in turn, is highly sensitive to changes in the operating conditions. Part I also outlines recent advances in mass transfer models for representing supercritical fluid extraction kinetics, which are essential for the scaling-up of one of the first-born but still very important areas of application and current commercialization, i.e., the extraction of solid vegetal raw matters.

Part II of the book is devoted to some of the most recent but not less successful technological developments in high pressure food technology, while Part III collects a wide number of highly promising applications in the field of food processing innovation.

The book is addressed primarily to graduate students and scientists involved in education and research in food engineering. Additionally, Part III of the book offers broad and comprehensive information on current applications of high pressure technology to develop special foods and food ingredients, particularly attractive to persons related to the food producing systems and food market.

This book is a result of the consorted efforts of a team of chemical engineers, chemists, and food technologists who have extensive experience in research and development of high pressure processes for innovation in food technology. It has been a privilege and an honor for the editors to work with them and they thank each and every one of the authors, experts from all around the world, for their outstanding contributions.

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Contents

Part I
Fundamentals

Chapter 1
High Pressure Phase Equilibria Measurement for Mixtures Comprising Food Substances

José M.S. Fonseca, Ralf Dohrn, and Stephanie Peper

1.1 High Pressure Phase Equilibria in the Food Industry

Over the last decades, different high-pressure processes have been gaining relevance in the food industry. One example is the so called high pressure processing, or pascalisation, a non-thermal equivalent to a pasteurisation. This process is based on the inactivation of microorganisms and enzymes present in food products through the application of high pressures, in the order of 600 MPa. By avoiding exposure of the food products to high temperatures, their natural qualities are better preserved. Polyphenol oxidase for example, is an enzyme present in all higher plants which causes undesirable colour modifications in fresh-cut fruit and in vegetable products and juices. High pressure inactivation of this enzyme has been studied in several natural products like avocados (Weemaes et al. 1998), grapes (Rapeanu et al. 2006), bananas (Rapeanu et al. 2006; Macdonald and Schaschke 2000), strawberries (Cano et al. 1997) and apple juices (Bayindirli et al. 2006; Buckow et al. 2009). A further "cold pasteurization method" of liquid food products is high pressure CO_2 treatment, a process reviewed for example by Spilimbergo and Bertucco (2003) and by Damar and Murat (2006). In case of high pressure carbon dioxide pasteurization, the microbial inactivation depends of the solubility of CO_2 in the liquid. Accurate phase equilibria data, in particular the solubility of CO_2 in liquid food products is therefore crucial for a better understanding and optimisation of the process.

In addition to food preservation, the texture of food products such as meat, fish and dairy products can be influenced by high pressure processing. Information on the modification of food protein functionality by high pressures can be found for example in the work of Messens et al. (1997). Many food manufacturing processes

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are performed to create a designed consistence for the product, in order to achieve a defined flavour perception and taste experience. Desired microstructures have to be created, in the form of phase separated networks which result in the desired characteristics. Among these microstructures are ice and fat crystals, oil and aqueous solutions of biopolymer droplets, and air bubbles. The creation of stable food products requires a comprehensive knowledge of the phase equilibria between the various components present in the product.

Another significant application is supercritical fluid extraction (SFE). This innovative, clean and environmental friendly technology is currently used in countless processes. For example in the production of extracts from hops, fruits, spices, nuts, and other natural materials, extraction of bioactive compounds from plants and herbs, the extraction of aromas, flavours and oils. But also for the processing of food products, such as in the decaffeination of coffee and tea, the defatting of cacao or of potato ships, rice cleaning, dealcoholisation of beverages, among other applications (Brunner 2005; Fornari et al. 2012).

Supercritical fluids present unique characteristics which make them excellent solvents. They usually show a relatively high density and consequently, a high solvation power. They present a relatively high selectivity, and have properties such as low viscosities and high diffusion coefficients which allow high extraction rates (Brunner 2005). Propane, ethane and CO_2, among others, can be used as supercritical solvent for extractions, but CO_2 is preferred due to its low critical temperature (of 304.1 K), its low cost, and its availability in purified form. Furthermore, CO_2 is inert, non-flammable, non-toxic, and it is easily removed from the extract.

The low critical temperature and a moderate critical pressure (7.38 MPa) are important aspects, as they allow the extraction processes to be performed at moderate temperatures and pressures, avoiding thermal degradation of natural compounds. The lower temperature needed for the extraction process is one of the advantages of the supercritical technology when compared to the classical approaches to recover plant extracts, which include steam-distillation or hydro-distillation. Furthermore, supercritical extracts have often been regarded as presenting a higher quality than those produced by the classical methods of hydro-distillation or liquid–solid extraction (Fornari et al. 2012). But carbon dioxide also presents some limitations. Being non-polar, high molecular weight or hydrophilic molecules are very often insoluble in supercritical CO_2. The solution for this is to use a polar co-solvent, such as ethanol, methanol, or ethyl acetate, among other possibilities.

As for many other processes in industry, phase equilibrium data, and in particular solubility data, are the basis for the design and optimization of the extraction process. This justifies the large number of publications which can be found in literature focusing on phase equilibria in systems containing food substances. In two recent reviews focusing on high-pressure phase equilibria results published between 2000 and 2008 (Dohrn et al. 2010; Fonseca et al. 2011a), around 10 % of all the (4,465) systems reviewed comprised food related substances.

Most of the high-pressure phase equilibria studies related to the food industry which can be found in literature focus on the determination of solubilities of solid or

liquid substances in supercritical CO_2, with or without co-solvents. Solubilities in other supercritical fluids can also be found, such as in ethane or propane (Chuang and Johannsen 2011; Schwarz et al. 2011). Less frequently, mixtures of supercritical fluids (Hegel et al. 2009) have also been used, as well as compressed water (Briones et al. 1994; Miller and Hawthorne 2000). Studies comprising solubility data of food substances in other compounds such as sulphur hexafluoride or different refrigerants like trifluoromethane (HFC-23), 1,1,1,3,3,3-hexafluoropropane (HFC-236fa), or 1,1,1,2-tetrafluoroethane (HFC-134a) (Knez et al. 2007) have also been published.

But the interest in phase equilibrium data goes beyond the knowledge of the solubility values in supercritical or in pressurised solvents. The determination of compositions of the two phases involved in the equilibrium, including therefore the solubility of the supercritical or compressed fluid in the food substances, is important for different reasons. The knowledge of the complete equilibrium data forms a better basis for the testing of thermodynamic models or equations of state (Hernández et al. 2010). Furthermore, CO_2 can be used as reaction medium for enzymatic reactions, and the information on the phase equilibria is crucial for the selection of the optimal conditions (Hernández et al. 2008).

More recently, new tendencies towards the use of green solvents have been observed. Compounds such as ethyl lactate can be used as a co-solvent in supercritical extractions with CO_2 or as solvent for chemical reactions (Villanueva Bermejo et al. 2013). It can also be used as a solvent in accelerated solvent extraction (Bermejo et al. 2013).

Other type of high-pressure phase equilibria studies can also be found in literature, equally related to the food industry, but dealing with indirect food applications, such as packaging, etc. Peters and co-workers (2012) for example have focused their attention into the pressure increase in packages of desserts containing carbon dioxide clathrate hydrates, used in the product to provide a strong perception of carbonation to the consumer. Heat shocks can lead to hydrate dissociation which could lead to a significant increase in the pressure inside the package. Another example is the work of Martín et al. (2009) focusing on the phase equilibria of CO_2 mixtures with water and polymers, in a study with potential applications in the improvement of food product preservation.

1.2 Experimental Methods for the Study of High-Pressure Phase Equilibria

The existence of a wide variety of methods for phase equilibria studies at high pressure is related to the fact that there is not a single method that is suitable for all the different types of measurements. In some cases it is not even possible to assess which method is the most appropriate for a specific determination, with different methods presenting both advantages and disadvantages. Before looking at which

methods are most commonly used and which methods have potential to be used more often in studies involving food products, it is helpful to make a categorisation of the experimental methods available.

The categorisation of the methods is not consensual, with different classifications proposed by different authors. Sometimes the same names are used for completely different methods. Expressions such as "static" and "dynamic" are often used with different meanings by different authors, creating further confusion. For example, the determination of the solubility of a solid substance in a supercritical or pressurised fluid can be determined by continuously passing the fluid through an equilibrium cell containing a matrix with the solute, a method frequently used in systems involving food substances. The fluid flows out of the equilibrium cell, saturated with the solute, and can be subsequently analysed for the solute concentration for the calculation of the solubility value. In another method, the equilibrium between two phases is studied in a closed cell using no sampling, relying on mass balances. To accelerate the equilibration of the system, one phase is constantly recirculated through the other. These two methods differ greatly from one another, from the experimental requirements, the experimental procedure, and the error sources which affect each of the methods. Nevertheless, they have both been classified as "dynamic" in literature. Also ebulliometry, in which the boiling temperature of a mixture is measured under isobaric conditions, is often referred by some authors as "dynamic VLE method".

To make matters more complex, the classification of a specific apparatus is not always straightforward, since combinations of different techniques may be used in different methods. Furthermore, some apparatus can be used with different methods, sometimes simultaneously.

The nomenclature adopted in this work and represented schematically in Fig. 1.1, tries to avoid the use of ambiguous denominations such as "static" or "dynamic", and it is similar to that recently presented in several publications (Dohrn et al. 2010, 2012; Fonseca et al. 2011a), consisting in the refinement of the classification used in previous reviews of the same series (Christov and Dohrn 2002; Dohrn and Brunner 1995; Fornari et al. 1990). Two fundamental classes of methods are considered, analytical and synthetic methods, depending on whether the composition of the phases in equilibrium are determined analytically or the system under study has a precisely known global composition, prepared (synthesised) previously to the experiment. In the case of analytical methods, the analysis of the phases in equilibrium can be performed with sampling and subsequent analysis, or without sampling, analysing the composition of the phases directly in the interior of the equilibrium cell, through the use of spectroscopic methods for example. As for the synthetic methods, a distinction can be made between experiments in which a phase change is observed, either the disappearance of an existing phase or the appearance of a new one, and experiments where no phase change occurs.

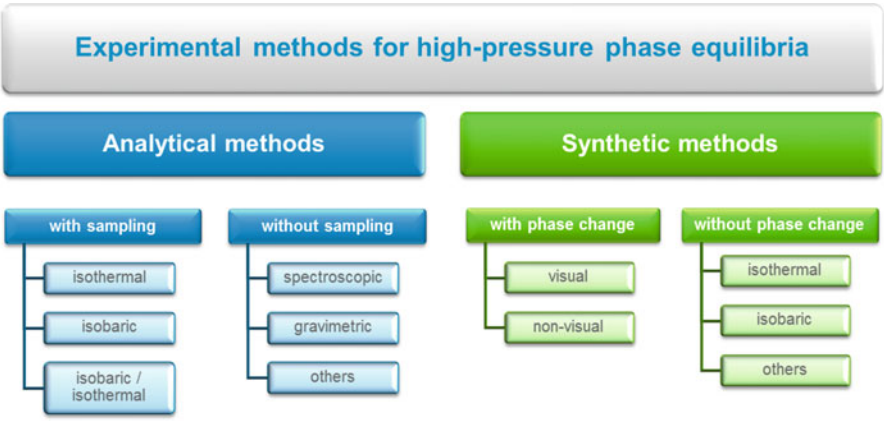

Fig. 1.1 Categorisation of the experimental methods available for the measurement of high-pressure phase equilibria

1.2.1 Analytical Methods

Analytical methods for the determination of high-pressure phase equilibria involve the determination of the compositions of the coexisting phases. In these methods the equilibrium cell is loaded with the components of the system to be studied without precise information of the total composition of the system, and the experimental conditions (temperature and pressure) are set. Once the equilibrium is achieved, the composition of the different phases can be determined by withdrawing a sample from the equilibrium cell for subsequent analysis, or by applying a suitable *in situ* physicochemical technique for analysis of the phases directly inside of the equilibrium cell, under pressure.

The main advantage of analytical methods is the possibility of application to multi-component systems without significant complications. They can also be applied where synthetic methods are disadvantageous, for example in cases in which phase boundaries depend strongly on the composition, or in the study of systems in which the interface is not easily observed. When compared to synthetic methods, the drawback of the analytical methods is the necessary higher complexity of the apparatus and experimental procedure, which must include an analytical part, often requiring time-consuming calibrations and prior optimisations of the analytical technique(s).

One of the most important aspects to take into account in the use of these methods is the possibility for the occurrence of significant pressure drop when the samples are withdrawn. This subject has been the object of a recent publication (Peper and Dohrn 2012). There are however several techniques which can be used in order to avoid or reduce this issue. Using a variable volume cell is probably one of the most common solutions to deal with this problem. Variable volume cells have a wide application range, not only in analytical methods, but also in cells from

where no sampling is performed (synthetic methods), readily allowing for isothermal changes in the pressure to promote the disappearance of phases, the appearance of new ones, or the observation of critical phenomena, for example. The variation in the volume of the cell is usually made by using a solid piston, whose position can be regulated either by direct mechanical actuation, or through a pressure transmitter medium and a syringe pump. This last technique can be especially efficient and practical if an electronic syringe pump is used (Fonseca and von Solms 2012). Another possibility is the use of a "liquid piston", using mercury that can be pumped into or out of the cell in order to change the volume available for the system under study (Shariati and Peters 2002). This solution has the advantage of being "leak-proof", but it also has some drawbacks, such as the toxicity of the metal and the possibility of reaction or solubilisation of components of the system being studied. Another possible solution to avoid pressure drop is to use a buffer volume in combination with a syringe pump, or by blocking off the content of the cell from the sample before withdrawing it.

Pressure drops are directly related to the proportion between the total volume of the cell in which the system is in equilibrium and the volume of the withdrawn sample. It is therefore possible to minimise the problem by increasing the volume of the cell. Cells with internal volumes of up to 9 dm^3 have been reported in literature (Gozalpour et al. 2003). The other obvious solution is, of course, to reduce the volume of the samples, and this can be achieved in several ways. A relatively common solution is the use of special valves, such as HPLC valves. These valves allow the collection of the sample into a loop of a previously selected volume, and by actuation of the valve, insert the sample directly into the carrier gas flow of a gas chromatograph, for example. Another possibility is to perform the sampling through capillaries. However, sampling through capillaries can lead to differential vaporization, leading to a scattering in the results, especially in the case of mixtures composed of both light and heavy components, caused by a pressure drop all along the capillary (Brunner et al. 1994). This problem can be circumvented by means of an adequate experimental design, ensuring that most of the pressure drop occurs at the end of the capillary close to the chromatographic circuit.

Researchers at the CENERG-TEP laboratory of the ENSMP (École Nationale-Supérieure des Mines de Paris), developed a system where a micro-stem ending with a nose, enters inside the capillary to reduce the cross-sectional area at the end of the capillary. The system is associated to a fast-acting pneumatic or electromagnetic valve, the ROLSI™ sampler (Rapid On-Line Sampler Injector) (Guilbot et al. 2000). These sampler-injectors were developed specifically for application in studies of phase equilibrium, with special attention to relevant details, making them a reference in the area, currently being used worldwide. Figure 1.2 depicts an electromagnetic ROLSI™ sampler-injector, used in a recently developed apparatus (Fonseca and von Solms 2012).

When special valves are used, the equilibrium cell can be coupled with analytical equipment, such as a high-performance liquid chromatograph, a supercritical fluid chromatograph, or in the most common solution, a gas chromatograph, allowing a sample of very small volume to be withdrawn and injected directly

Fig. 1.2 Picture of an electromagnetic ROLSI™ sampler-injector, showing the actuator and the capillary through which sampling is made

into a carrier gas flow, without any manipulation. Both in the case of the HPLC-valves and of the ROLSI™ samplers, the reduced sample volume simplifies the analytical processes, making possible the analysis of the whole sample by chromatography, without the need for dilutions and without risk of saturation of the columns. Care should be taken when studying systems containing solid particles, which could lead to a blockage of the capillaries.

In order to simplify the experimental apparatus, sometimes only a single sampling valve is used in the equilibrium cell, associated to a mechanism that allows the sampler to move, allowing sampling from different phases. An example of such is the work of Laursen et al. (2002), who used a high pressure view-cell equipped with a movable sampling needle in the study of VLLE systems. This apparatus was eventually decommissioned and the cell recuperated for a new experimental set-up using a different method (Fonseca and von Solms 2014). Reports of a cell equipped with a moveable ROLSI™ sampler can also be found in the literature (Richon 2009). Care should be taken that the movement of the sampler does not influence significantly the pressure in the cell, as this usually leads to a change in the internal volume of the cell. This is especially relevant for cells of reduced dimensions. Another point to be considered, is that the movement of the sampler form one phase to the other may lead to disturbances in the interface, which in systems of difficult phase separation can constitute a serious problem.

The methods in which samples are withdrawn from the equilibrium cell can be classified, depending on the procedure used in the achievement of the equilibrium, into isothermal methods, isobaric methods and isobaric-isothermal methods. These methods are notable for allowing a better understanding of the equilibrium systems under study, with the desirable characterisation of all the different phases involved (Fonseca 2010).

Another approach for circumventing the problem of the pressure drop associated with sampling processes is not to carry out any sampling, performing an analysis *in situ* through the use of a convenient physicochemical method, most commonly with spectroscopic methods. In this type of methods, the equilibrium cell or at least part of it should be transparent to the type of radiation used. The use of sapphire windows is a frequent choice, due to the good transmission characteristics over the

visible, near IR and near UV spectrum of this material, with a useful optical transmission range of wavelengths ranging from 200 to 5,500 nm (Weber 2002; Gervais 1998). A disadvantage of these methods of analysis is that occasionally, only the determination of the concentration of a particular compound in the different phases is possible, which may be a limiting factor in the study of multi-component systems. Another aspect to consider is the requirement for time-consuming spectroscopic calibrations under different experimental conditions, including different pressures.

Irrespective of the experimental method applied, the use of windows, view-ports, or entire segments of the equilibrium cell made of transparent materials as a 360° window (Shariati and Peters 2002; Folas et al. 2007a), also allows a visual observation of the contents of the cell, making possible to identify interfaces between phases as well as their volume, among other phenomena such as critical points, foaming, etc. These windows can be made of thick glass or quartz, but most commonly sapphire is used. Although more expensive than other options, sapphire is one of the hardest materials, being much stronger than glass and characterised by a good chemical resistance, thermal conductivity and thermal stability, making it ideal for high-pressure applications.

Analytical Isothermal Methods. In isothermal methods, the temperature of the system is kept constant during the whole experiment, including throughout the equilibration process, while other equilibrium properties such as the pressure and the composition of the phases vary with time, until the achievement of the equilibrium values. The experiment starts by loading the equilibrium cell with the substances of interest. The initial value for the pressure is set under or above the desired value, taking into consideration the effect that the equilibration process will have on its value. Once the system is close to equilibrium, further adjustments in the pressure can be performed, by adding or withdrawing material, followed by a new equilibration process. When in equilibrium, the pressure reaches a stable value, and usually, additional time is given to the system without stirring, rocking or recirculation, before withdrawing any samples or performing any measurements. This is done in order to guarantee a good separation of the different phases, and to avoid the occurrence of non-homogeneous samples, where the sampled phase still contains material from another phase(s), such as droplets, bubbles or solid particles (Secuianu et al. 2003). Figure 1.3 summarises these three fundamental steps, characteristic of the analytical isothermal methods.

In order to reduce the time necessary for the equilibration of the system, several techniques can be employed to promote the contact between the different phases, increasing the efficiency of the mass transfer. This is usually done by rocking the cell, recirculating one or more phases, or most commonly, by stirring the mixture. Stirring can be done using with a motor-driven rotating axis passing through the wall of the cell, making this an especially sensitive point for the occurrence of leaks, due to the wearing of the seals. This can constitute a serious problem since the leak-induced pressure prevents a complete equilibration of the system. Alternatively, stirring can be promoted through the use of a magnetic system that although less efficient under certain conditions, is simpler and does not interfere

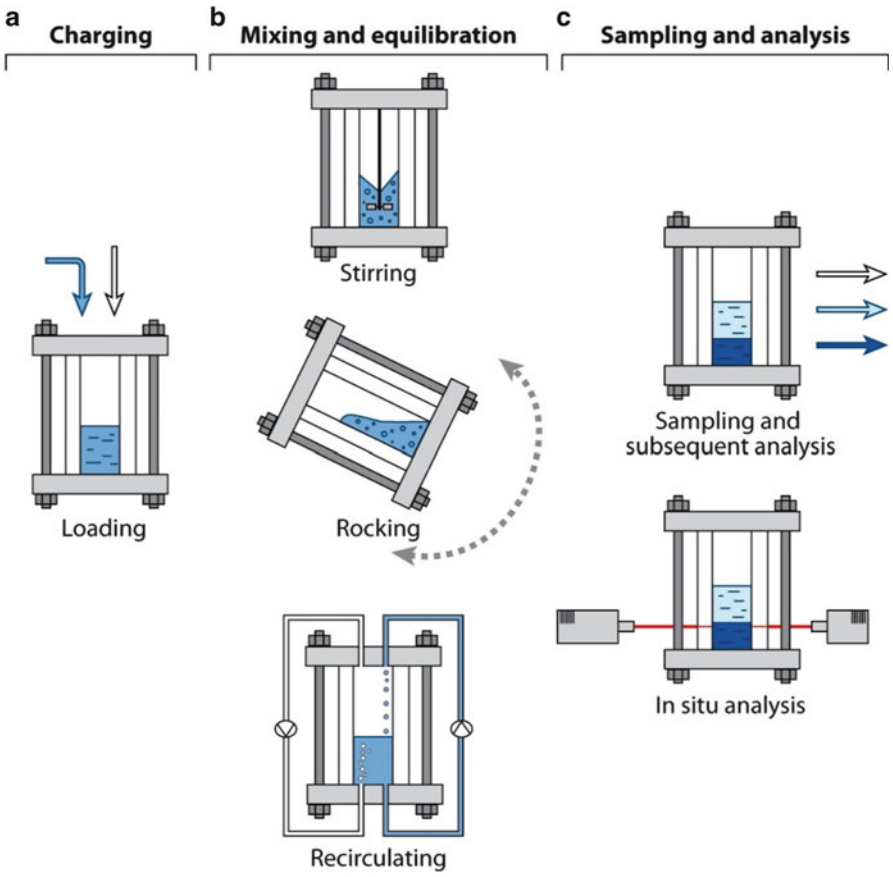

Fig. 1.3 Schematic diagram showing the three fundamental steps in the application of analytical isothermal methods (Dohrn et al. 2012)

with the air tightness of the cell. The efficiency of magnetic stirring can be inadequate when the phases to be mixed have a high viscosity, or in the case of high temperature applications, as magnets tend to lose their strength with the increase of temperature. This last issue can be minimised by the use of a cooled magnetic stirring system, although this may have a negative impact on the homogeneity of the temperature of the equilibrium cell. In low-temperature applications, care should also be taken so that the heat usually produced by the stirring motor does not interfere with the temperature of the equilibrium cell (Fonseca and von Solms 2012). In the study of systems with gas hydrates, or systems involving very viscous phases, rocking the equilibrium cell can be a good answer to promote a faster achievement of the equilibrium (Draucker et al. 2006; Mesiano et al. 2001; Ostergaard et al. 2000). It is an important requirement for this that all the

connections to the cell are flexible. These methods where stirring or rocking of the cell is performed are sometimes designated as "static-analytical method" by some authors.

Another common technique is to promote the recirculation of one of the phases, by means of magnetic pumps, forcing the gas phase to be continuously bubbled through the liquid phase (Fedele et al. 2007; Jou and Mather 2007), or pumping the liquid phase to the top of the cell, where it re-enters through the gas phase (Brass et al. 2000; Marcelino Neto and Barbosa 2008). Recirculation of both phases is also used by several authors (Kato et al. 2006; Kodama et al. 2007; Missopolinou et al. 2005; Tsivintzelis et al. 2004). This is a very effective method for expediting the equilibration process, although the further complexity in terms of connections and tubing can represent a problem, associated with the occurrence of pressure leaks. An additional aspect to take into account is the requirement for a good quality pump, characterised by a small pressure drop, and the need for a uniform temperature throughout the whole loop, so as to avoid partial condensation or vaporization in the recirculation line(s). Consequently, the application of recirculation methods is not recommended for studies in the region close to the critical point, where small changes in temperature and pressure have a strong influence on the phase behaviour (Nagahama 1996). Another drawback in the use of recirculation methods is the cost associated with good quality pumps.

Notwithstanding these aspects, recirculation methods may present advantages when conveniently combined with specific sampling procedures, allowing for example the isobaric filling of a sample volume. Samples can be withdrawn by placing a sampling valve in the recirculation line (Wagner et al. 2000), or by blocking off a determined volume between two valves in the recirculation loop (Marcelino Neto and Barbosa 2008; Kim et al. 2000).

Another possibility in the use of recirculation methods is the inclusion in the recirculation loop(s) of a vibrating-tube densimeter allowing the density of the circulated phase to be determined very easily (Missopolinou et al. 2005; Tsivintzelis et al. 2004; Kato et al. 2007). Figure 1.4 presents a schematic drawing of the apparatus used by Tsivintzelis et al. (Missopolinou et al. 2005; Tsivintzelis et al. 2004), where both recirculation loops have been highlighted for easier visual identification. In such density measurements, it is important that the recirculation pumps are turned off during the density measurements, in order to avoid errors due to pulsation (Freitag et al. 2004).

Recirculation methods are sometimes called "dynamic" methods by some authors, despite having almost everything in common with the methods sometimes denominated "static-analytical method". This illustrates once again the ambiguity of the expressions "static" and "dynamic".

In a particular sampling technique, the sampling volume is kept within the equilibrium cell. This method can be used for example in the measurement of the solubility of solids in supercritical fluids. Sherman et al. (2000) placed an excess amount of solute in a glass vial, capped with a coarse filter paper, in the equilibrium cell. After equilibration and careful depressurization, the vial is removed and weighed. The solubility can be calculated from the difference of the initial and

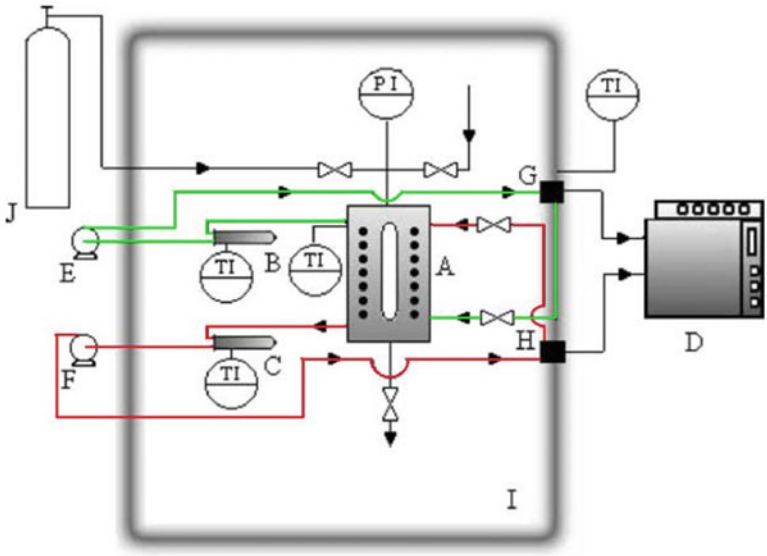

Fig. 1.4 Schematic drawing of the apparatus used by Tsivintzelis et al. (Missopolinou et al. 2005; Tsivintzelis et al. 2004). The recirculation loops for the liquid and the gas phase, containing the vibrating densimeters marked by *B* and *C*, were highlighted for easier identification

final mass of the solute in the vial and the relation between the volume of the vial and that of the equilibrium cell. As an alternative, Galia et al. (2002) used three vials, of which only one was initially filled with the solute.

Nikitin et al. (2003a, b) used an alternative technique for measuring sorption of carbon dioxide in polystyrene, avoiding sampling from a high-pressure cell.

The polymer is first placed in the equilibrium cell, which is then pressurised up to the desired value. For several hours, the volatile component is absorbed in the polymer, until equilibrium is achieved, Next, the authors perform a fast depressurisation (in less than 10 s), followed by a quick transport of the sample to an analytical balance (in less than 5 s). Once the sample is placed in the analytical balance, the decreasing of the mass of the polymer sample due to desorption of carbon dioxide is recorded over time, and the initial value of sorbate mass can be determined by extrapolation to the beginning of the depressurization process.

In the analytical isothermal methods, the equilibration process can be followed over time, and sampling is only performed after equilibrium is achieved, which usually is assumed by observing a constant pressure in the cell. This possibility is an advantage when compared with analytical isobaric-isothermal methods, where there is the risk of an incomplete equilibration process if the experimental conditions are not properly chosen. However, it should be considered that a constant pressure is not always a clear indicator that the equilibration has been achieved. In the study of multiphase systems, such as vapour-liquid-liquid equilibrium (VLLE) for example, the pressure may present an apparently stable value with time, even though equilibrium in the cell has not been achieved. The reason for this is that the

mass transfer between the two liquid phases can have very little influence on the total pressure of the system (Fonseca 2010). In the study of such systems, equilibration should be confirmed by the sample and analysis of the composition of the liquid phases, at different points in time. Special attention should be given to the low concentrations in the cases of minor components.

Analytical Isobaric-Isothermal Methods. In isobaric-isothermal methods, sometimes called "dynamic methods", one or more fluid streams are continuously pumped into a thermostated equilibrium cell. The pressure is kept constant during the whole experiment by controlling an effluent stream, most commonly the vapour phase, using a back-pressure regulator. One of the most important aspects to take into consideration in the application of these methods is related to the time needed for the full equilibration of the system to be attained, which should be sufficiently short, and imposes limitations on the velocity of the flows. If the equilibration is too slow, and/or the flow rate(s) too high, there is the risk that the effluents will not correspond to the equilibrium state as desired. Previously to any set of experiments, it is recommended to carry out a number of tests under different flow conditions, in order to evaluate the limits for the systems under study.

Continuous-flow Method. In a typical design of a continuous-flow method, shown schematically in Fig. 1.5, high-pressure metering pumps are used to supply a constant flow of the components, which after a pre-heating stage enter a mixer kept at a desired temperature, where the phase equilibrium is attained. Often, static mixers are used (Fonseca et al. 2003; Ruivo et al. 2004). The feed stream from the mixer is then separated in an equilibrium cell into a vapour and a liquid phase. To facilitate phase separation, a cyclone separator was used by Fonseca et al. (2003) for example. Normally, effluents from both phases are continuously withdrawn, depressurized, accumulated and analysed only after the experiment has been concluded. Nevertheless, on-line analysis is also sometimes possible. Hurst et al. (2002) describe a continuous-flow cell equipped with large diameter optical ports suitable for visual observation, and for Raman spectroscopic studies of aqueous solutions at temperatures up to 770 K (500 °C). The pressure in the system is adjusted by means of a back-pressure regulator that controls the effluent stream of the vapour phase. The interface level between the fluid phases in the equilibrium cell can sometimes be determined visually (Bamberger et al. 2000), and adjusted with a bottom-phase expansion valve.

When compared with the isothermal methods described above, the continuous-flow methods present the important advantage that sampling and analysis can be done without disturbance of the equilibrium. Furthermore, if large samples are needed, for example to facilitate the analysis of traces of particular compounds in some phases, the run time of the experiment can simply be extended in order to accumulate more material, without the need for increasing the volume of the equilibrium cell or the dimensions of the experimental set-up. Another great advantage of this type of method is related to the short residence time of the components in the apparatus, reducing the possibility for thermal decomposition or polymerization reactions when performing measurements at higher temperatures (Mahmood et al. 2001). However the continuous-flow methods usually require the

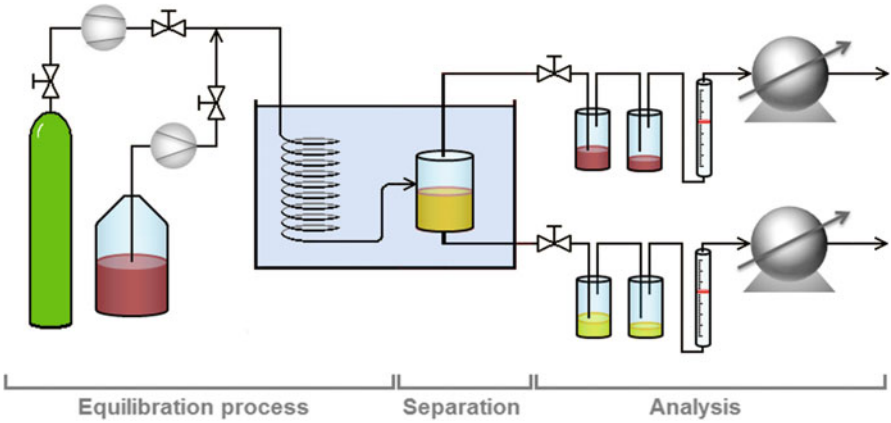

Fig. 1.5 Schematic diagram showing a typical design of a continuous-flow method, showing the three fundamental steps of the method

use of larger amounts of chemicals, which can be a disadvantage when compared with the isothermal analytical methods, especially when hazardous or expensive compounds are involved. Furthermore, the experimental procedure can be more complicated than for the isothermal analytical methods, due to the somewhat more complex process control involved.

Semi-flow Methods. In semi-flow methods, only one of the phases is flowing while the other is stationary in an equilibrium cell. Semi-flow methods can also be known as "single-pass flow methods", "gas-saturation methods" or "pure-gas circulation methods", depending on the authors. The principle of the method is very similar to that of a supercritical fluid extraction, in which a solid substrate is placed in a fixed bed, through which the supercritical fluid flows, extracting the substrate from its matrix. This similarity is likely to be one of the reasons why this method has been widely used in studies related to the food industry, in the determination of solubilities of different substances in supercritical fluids. The same apparatus can in principle be used for phase equilibria studies and in the optimisation of extraction processes at lab or pilot-plant scale.

Typically, in the semi-flow methods, a high-pressure stream of gas or of a supercritical fluid is passed through two cells connected in series, containing the low-boiling component in a condensed phase. The first cell is used as a pre-saturator while the second cell operates as an equilibrium cell. Upon equilibration, the effluent of the vapour phase is reduced in pressure and conducted through a trap, where the condensed component is collected. The quantity of gas can be determined volumetrically, by means of a gas meter, or using a flow meter such as a wet test meter (Cheng et al. 2001; Eustaquio-Rincón and Trejo 2001). Figure 1.6 presents a typical experimental set-up for the application of this method, with emphasis on the different steps of the measurement.

In most applications, only the composition of the vapour phase is analysed, for example in the determination of the solubility of a low-boiling (liquid or solid)

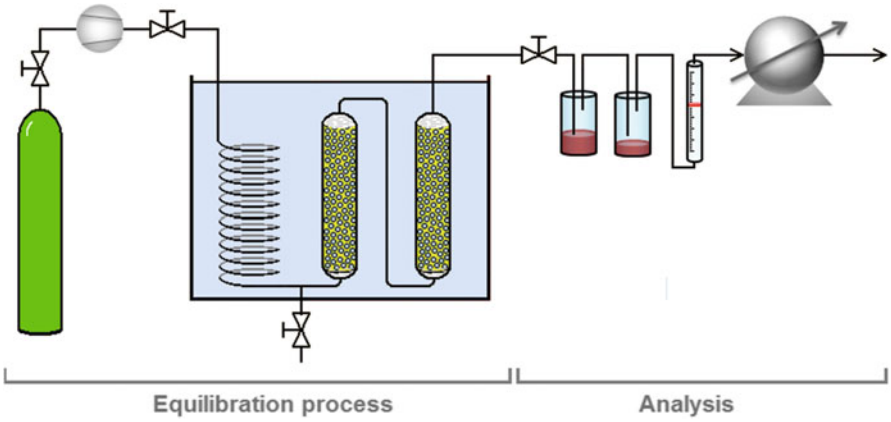

Fig. 1.6 Schematic diagram showing a typical design of a semi-flow method, demonstrating the fundamental steps of the method

substance in a supercritical gas (Berna et al. 2001; Cheng et al. 2002). A wide variety of techniques are suitable for the determination of the composition of the vapour-phase effluent, through the use of a spectroscopic method (Fu et al. 2007; Kordikowski et al. 2002), using a multi-port sampling valve and a subsequent HPLC analysis (Goodarznia and Esmaeilzadeh 2002), using an absorption bath after previous expansion to atmospheric pressure using cold traps (Eustaquio-Rincón and Trejo 2001), or by means of a chromatography column filled with an appropriate adsorbent for the solute under study (Alessi et al. 2003).

In this type of solubility measurement, no samples from the stationary condensed phase need to be taken. However, when a semi-flow method is used in VLE studies, the composition of the condensed phase needs to be determined as well. A sample from the liquid phase can simply be withdrawn through a common valve and tubing, and depressurized before further analysis (Cheng et al. 2000). Furthermore, semi-flow methods can also be used in the measurement of the solubility of a gas in a liquid, as in the work presented by Tan et al. (2001). The experimental procedure is similar to the one just described for the vapour-liquid equilibria studies, but without the need to determine the composition of the effluent from the vapour phase. Nonetheless, the analysis of the effluent from the vapour phase can also be used in the calculation of the solubility of the gas in a liquid. In studies related with CO_2 capture for example, the solubility of this gas in a condensed phase (an amine solution or an ionic liquid) can also be determined by passing a stream of a typical flue gas mixture of known composition through a solution, and continuously analysing the amount of carbon dioxide in the effluent flow. A typical experiment is characterised by an initial decrease in the amount of CO_2 in the effluent flow, as the gas gets absorbed in the liquid phase, followed by an increase back to its initial value, as the condensed phase becomes saturated with the gas (Lerche 2012).

Tuma et al. (2001) used a modified supercritical fluid chromatograph (SFC) to measure the solubility of dyes in carbon dioxide. The column was filled with finely pulverized dyestuff and the analysis of the vapour-phase stream was performed by means of Vis-spectroscopy.

Chromatographic Methods. In the chromatographic methods, the retention of a solute in a chromatographic column is measured, and related to the Gibbs energy of solute transfer between the stationary and the mobile phase. Roth (2004) presents a review on applications of supercritical fluid chromatography (SFC) for the determination of the relative values of solute solubilities in supercritical fluids, and on the determination of solute partition coefficients between a supercritical fluid and the stationary phase. In SFC, the thermodynamic analysis of solute retention is more challenging than in common gas chromatography since the uptake of the mobile phase fluid by the stationary phase is no longer negligible. The main advantage of the chromatographic methods is the possibility to determine equilibrium properties and diffusion coefficients simultaneously, in one experiment (Funazukuri et al. 2000).

Chester (2004) reviewed a chromatographic technique, which he calls "flow injection peak-shape method", that allows the determination of the pressure and temperature coordinates of the vapour-liquid critical locus of binary systems. This technique can be implemented using open-tubular SFC instrumentation, by replacing the SFC column with several meters of fused-silica tube. This tube may be deactivated but is not coated with a stationary phase. The procedure to map a critical locus involves selecting a temperature, then making injections at various pressures while looking for the pressure where the peaks change from their rectangular appearance (liquid phase and vapour phase present in the column) to distorted Gaussian (single homogeneous phase in the column). This transition pressure provides an estimate of the mixture critical pressure, corresponding to the oven temperature.

Analytical Isobaric Methods. One of the most common methods for the measurement of vapour pressures in the pressure range from 10 to 100 kPa is ebulliometry (from Latin *ebullio* "to boil, to bubble up") (Fonseca et al. 2011b). The method can however be extended to the studies at high pressures (Susial et al. 2010). In this method, the boiling temperature of a pure substance or mixture is measured under isobaric conditions and the phase compositions are determined after sampling and analysis. The experimental apparatus, an ebulliometer, is fundamentally a one-stage total-reflux boiler equipped with a vapour-lift pump to spray slugs of equilibrated liquid and vapour onto a thermometer well. As opposed to the more frequently used synthetic isobaric method described later in this chapter, vapour and liquid streams are separated, collected and analysed. The compositions of the liquid and the vapour phase vary with time, towards a stable value that should not differ significantly from the true equilibrium value.

Analytical Spectroscopic Methods. Spectroscopic methods allow the analysis of the phase composition at high pressures, without the need to withdraw any samples. A number of techniques can be used in these methods, such as near infrared spectroscopy (Haines et al. 2008), or the ^{2}H NMR technique combined with light

microscopy used by Cruz Francisco et al. (2004) in the study of the phase behaviour of lecithin + water + hydrocarbon + carbon dioxide mixtures. Pasquali et al. (2008) used attenuated total reflection infrared (ATR-IR) spectroscopy to simultaneously measure the sorption of CO_2 in polyethylene glycol (PEG) and the polymer swelling.

Rondinone et al. (2003) developed a single-crystal sapphire cell for performing neutron-scattering experiments on gas hydrates. Since the sapphire crystal only contains aluminium and oxygen, it possesses a low incoherent neutron scattering and absorption cross section, having a low contribution to the background signal. Shieh and co-workers (2004) studied the effect of carbon dioxide on the morphological structure of compatible crystalline/amorphous polymer blends by means of small angle X-ray scattering (SAXS) with the measurement of absolute scattering intensity.

In the analytical spectroscopic methods, the advantage of avoiding the sampling process is often outweighed by the inherent disadvantages. In addition to the inability to perform a complete characterisation of the composition of the phases, as already mentioned, it is also necessary to consider the requirement of time consuming calibrations at various pressures.

Other methods, also using Raman spectroscopy for the detection of gas hydrates (Hashimoto et al. 2006; Jager and Sloan 2001) or laser scattering techniques (Najdanovic-Visak et al. 2003; Trindade et al. 2007), should not be included in the analytical spectroscopic methods, since these techniques are used in the detection of a new phase, rather than in the quantitative analysis of phase compositions. Such methods will be discussed later in this chapter.

Analytical Gravimetric Methods. Gravimetric methods are based in the monitoring of the mass of a non-volatile condensed phase, such as a polymer (Pantoula et al. 2007; von Solms et al. 2004) or an ionic liquid (Anthony et al. 2002), in phase equilibrium with a fluid phase. Using additional information, like the density of the phases, the phase compositions can be determined.

Palamara et al. (2003) placed an entire high-pressure equilibrium cell on a balance, and performed the equilibration under isobaric conditions. A very important aspect to consider in such application is the weight of the cell and the attached valve, since for commercially available analytical balances a higher sensitivity is synonymous with a lower maximum load capacity. In the study of Palamara et al. (2003), the cell and the attached valve have an approximate weight of only 190 g.

Cotugno et al. (2003) and Moore and Wanke (2001), placed a quartz spring balance and an electro microbalance, respectively, within a high-pressure cell, in order to measure the sorption of gases in polymers.

Kleinrahm and Wagner (1986) developed a unique balance, a so-called magnetic suspension balance, intended for accurate measurements of fluid densities, with the main advantage that both the sample and the balance are isolated. An electronically controlled magnetic suspension coupling is used to transmit the measured force from the sample enclosed in a pressure vessel to a microbalance. The suspension magnet, which is used for transmitting the force, consists of a permanent magnet, a

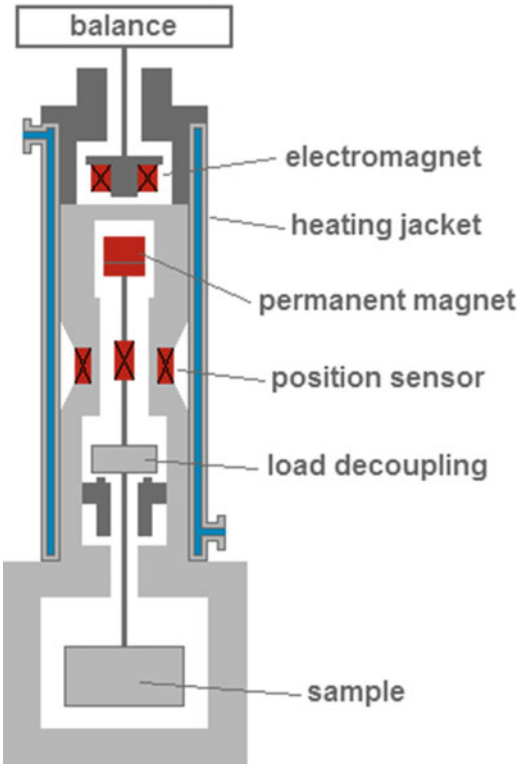

Fig. 1.7 Schematic representation of a magnetic suspension balance

sensor core and a device for decoupling the measuring-load. An electromagnet attached at the under-floor weighing hook of a balance, maintains the freely suspended state of the suspension magnet by means of an electronic control unit. Using this magnetic suspension coupling, the measuring force is transmitted without contact from the measuring chamber to the microbalance, located outside the chamber under ambient atmospheric conditions. To better illustrate this concept, a schematic diagram of a magnetic suspension balance is shown in Fig. 1.7.

Several researchers used a magnetic suspension balance to measure the solubility and diffusivity of volatile components in polymers, such as the case of Sato et al. (2001).

Gravimetric methods require corrections for buoyancy effects and, consequently, the exact information on the density of the fluid phase and on the density and volume of the condensed phase is essential, particularly at high pressures.

Other Analytical Methods. Quartz crystal microbalances can be used in the study of phase equilibria using a different principle. These instruments are usually based on the piezoelectric effect observed in an AT-cut quartz crystal. The crystal under the influence of an applied alternating electric voltage undergoes a shear deformation, with a maximum at a specific frequency known as the resonance frequency (Boudouris et al. 2001). This resonance frequency is dependent on the

mass, and thus any mass change will result in an associated frequency shift. Sorption or solubility experiments are based in the measurement of the resonance frequency of the bare (clean) crystal, of the same crystal coated with the substance of interest, and finally, of the coated crystal after the equilibrium between the substance in the surface of the crystal and the gas or supercritical fluid is attained, all at the same controlled temperature. The resonance frequency of a reference crystal can also be measured at the same conditions of the experiments, in order to compensate any temperature or pressure effects.

Park et al. (2004) examined the effect of temperature deviation and pressure change on the frequency shift by measuring the frequency change of an uncoated crystal under high-pressure carbon dioxide. Further developments to the quartz crystal technique, were performed by Guigard et al. (2001), who applied the method in the measurement of low solubilities in supercritical fluids. A small mass of solute is deposited on the crystal and the solubility is measured by observing the change in the frequency of the crystal as the solute dissolves in the supercritical fluid.

As an example of how the same apparatus can be used in the application of different methods, Mohammadi et al. (2003) used a quartz crystal balance as an extremely sensitive detector for the appearance of hydrates, where a change of mass of merely 1 ng resulted in a change of frequency of 1 Hz. Since the balance is used merely for detecting the appearance of a new phase, the method is not considered to be analytical method, and should be included in the category of the synthetic methods, discussed later in this chapter.

When compared to other methods, the quartz crystal microbalance provides a much higher sensitivity in the determination of mass changes, meaning that smaller samples are necessary to perform the experiments, which in turn accounts for a faster equilibration process and faster experiments (Oliveira et al. 2004). Nevertheless, the quartz crystal microbalance technique also has some drawbacks. The preparation and loading of the sample onto the crystal can be very challenging, and the system tends to be highly sensitive to small changes in electrical current.

Among other methods, less frequently used, Abbott et al. (2002) presented a capacitive (relative permittivity) method, in the measurement of the solubility of low-volatile substances in supercritical gases. The authors used a 25 cm^3 high-pressure cell, lined with a layer of Teflon. A capacitor consisting of two parallel rectangular stainless steel plates, with an area of 6.6 cm^2 and held 1 mm apart by Teflon spacers was placed in the fluid phase. The dielectric constant of the saturated vapour phase was measured at different pressures. In order to calculate the concentration of the solute in the vapour phase from the dielectric constant, the permanent dipole moments and the molecular polarizabilites of the different components of the mixture need to be known.

1.2.2 Synthetic Methods

The idea behind the synthetic methods is again to avoid the need for sampling, but this time by using a mixture with a precisely known composition, and subsequently observing its phase behaviour in an equilibrium cell, measuring only properties such as pressure and temperature in the equilibrium state. Synthetic methods can be based on a phase transition, where the disappearance or the appearance of a new phase is detected, or not. But in both cases, a mixture with a precisely known composition has to be prepared (synthesised), and the challenge of analysing fluid mixtures is substituted by the challenge of carefully preparing them.

Equilibrium cells for synthetic methods can in principle be smaller than cells used with analytical methods. Since no sampling is necessary, there is no need for a large volume equilibrium cell to minimize the pressure drops generated by the sampling procedure. But also for synthetic methods, a larger cell volume can, in certain conditions, be advantageous (Fonseca and von Solms 2014).

In the case of synthetic methods with a phase transition, the initial conditions can for example, be selected in order to promote the existence of one single homogeneous phase in the system. During the experiment, the pressure and/or temperature conditions are altered, leading to the appearance of a new phase. These experiments can be used merely to know the pressure and temperature coordinates of a specific phase transition, but inferring the composition of one of the phases is also possible. The moment when the second phase appears, and while it is still very small, the composition of the large phase can be considered to be equal to the global composition of the system, each experiment yielding one point of the pTx phase envelope. The detection of a phase transition can be done by visual means, in which case the methods are classified as visual synthetic methods, or non-visually, in the case of non-visual synthetic methods.

Not only variations on pressure and temperature can be used to promote the phase transition. This effect can also be achieved by a change in the overall composition of the system, as in the work presented by Wubbolts et al. (2004) who used a method sometimes designated as "vanishing-point method" or "clear-point method", in the study of solid–liquid equilibrium. In this method, a clear solution of a given solute concentration is added to a known amount of anti-solvent until the last crystal disappears. The composition of the mixture at this vanishing point corresponds to the solubility of the mixture. Repeating the procedure with solutions of different concentrations leads to additional points for the solubility curve.

The application of synthetic methods without the occurrence of a phase transition is dependent on the knowledge of a number of equilibrium properties, such as pressure, temperature, phase volumes and densities, which are subsequently used in calculations involving material balances for the characterisation of the phase compositions. These methods can be divided in isothermal, isobaric and other methods.

Synthetic methods with a phase transition have been by far more common than synthetic methods without a phase transition. This is especially true for studies of systems related to the food industry. But in other areas of application the use synthetic methods without a phase transition have been going through an expansion over the last years, being widely used for example in the determination of gas solubilities in low-volatility condensed phases, for example with application on CO_2 capture.

According to recent reviews (Dohrn et al. 2010; Fonseca et al. 2011a), in the period of 2000–2008, 58.3 % of the 4,465 systems considered were studied through a synthetic method. Considering only the period from 2005 to 2008, synthetic methods accounted for 63 %, almost two thirds, of the reviewed systems. As shown later in this chapter, the situation is significantly different though, when studies related to the food industry are considered.

In general, synthetic methods can be used where the application of analytical methods becomes more problematic, as in situations where phase separation is difficult due to similar densities of the coexisting phases, or near or even at critical points. For this reason, Kodama and his co-workers (Kodama et al. 2007) recently presented a study on the phase equilibrium of the binary system ethylene + butanol, in which they used an analytical method for the majority of the measurements, but a synthetic method for determinations near the critical region.

Often, the experimental procedure is easy and quick (Schneider 1975), and since no sampling is required, the experimental set-up can be much simpler and rather inexpensive, without the sampling and the analytical equipment (Fonseca and von Solms 2014). The equilibrium cell can be more compact, of a smaller volume, as there are no pressure drop problems associated with sampling, allowing also the development of equipment suitable for extreme conditions regarding temperature and pressure (Manara et al. 2002). Cohen-Adad (2001) describes a diamond anvil cell that can be used for pressures up to 135 GPa. Smith and Fang (2009) recently presented a review on the use of this type of cells, emphasising the advantages of this technique for application at high pressure and high temperature conditions. According to the authors, the very small volume of this type of cell also facilitates the study of supercritical systems at high density, from 400 to 1200 $kg \cdot m^{-3}$, which can otherwise be difficult or expensive with other methods. In this type of cells, the two diamond anvils are forced together by means of spring-loaded screws or using a standard arm-lever block. The diamond anvils are cemented in place through a rigidly mounted sample gasket. The sample is placed inside the gasket hole, together with a pressure transmitting fluid and small ruby chips which are using to measure the pressure by excitation of their fluorescence.

The limited information that synthetic methods can sometimes provide in the study of multi-component systems, for which the tie lines cannot be determined without additional experiments, constitutes perhaps the most important drawback of these methods. Furthermore, in some experiments the phase compositions in equilibrium are calculated based on approximations and rely on the results of equations of state or other prediction methods. Nevertheless, synthetic methods are still a powerful solution for the study of simpler systems.

Visual Synthetic Methods. In the most common of the synthetic methods, the appearance of a new phase is detected by visual observation of the resulting turbidity or the appearance of a meniscus in a view-cell. Limitations exist in the cases of isooptic systems, where the coexisting phases have approximately the same refractive index, making visual observation unfeasible. The visual synthetic method has a wide application range, and it can be used not only for the determination of simple vapour-liquid equilibria or solubilities, but also in the study of more complex phase behaviour, such as multi-phase equilibria (Franceschi et al. 2004), solid–liquid equilibria (Yang et al. 2002), critical curves of mixtures (Diefenbacher and Türk 2001), gas hydrate formation (Link et al. 2003), cloud-point determinations (Najdanovic-Visak et al. 2003) or phase equilibria in polymer-solvent systems (Byun and McHugh 2000).

The Cailletet apparatus of the recently discontinued high-pressure thermodynamics laboratory in TU Delft, The Netherlands (Shariati and Peters 2002), named after the French physicist and inventor Louis-Paul Cailletet (1832–1913), has been one of the most frequently used apparatus based on a synthetic visual method. It consists of a thick-walled Pyrex glass tube (500 mm long, 3 mm inner diameter) with the open end placed in an autoclave and immersed in mercury. The mercury confines the sample in the Cailletet tube and a stainless steel ball driven by reciprocating magnets provides the mixing of the system. Daridon et al. (2002) used a very small cell with a volume of only 0.03 cm^3, for the visual observation of synthetic waxes at high pressures, placed within a polarizing microscope, allowing the visual observation of crystals of 2 μm.

To improve the detection of phase transitions, some authors use special techniques such as laser light scattering (Najdanovic-Visak et al. 2003). Jager and Sloan (2001) used Raman spectroscopy in order to detect the appearance of gas hydrates, while Dong et al. (2002), made use of additional small angle X-ray scattering measurements in the determination of the median micelle size of the water in carbon dioxide micro emulsions.

These techniques are included in the category of "visual" methods as they depend normally on the use of a transparent material such as sapphire windows, etc. Synthetic visual methods are by far the most frequent type of method found in the literature according to recent reviews (Dohrn et al. 2010; Fonseca et al. 2011a), and have been used in the study of 35.8 % of the 4,465 systems covered by these reviews, in the period between 2000 and 2008.

Non-Visual Synthetic Methods. As an alternative to visual inspection, for example when using all-metal cells, other physical properties can be monitored in order to detect the occurrence of phase transitions. Minicucci et al. (2002) for example, made use of transmitted X-rays as the basis for phase detection, while Drozd-Rzoska et al. (2004) used measurements of the relative dielectric permittivity in liquid-liquid equilibrium measurements.

In cases where the volume of a variable-volume cell can be known accurately at any instant, the appearance of a new phase can be obtained from the abrupt change in slope on the pressure-volume plot, sometimes more accurately than by visual

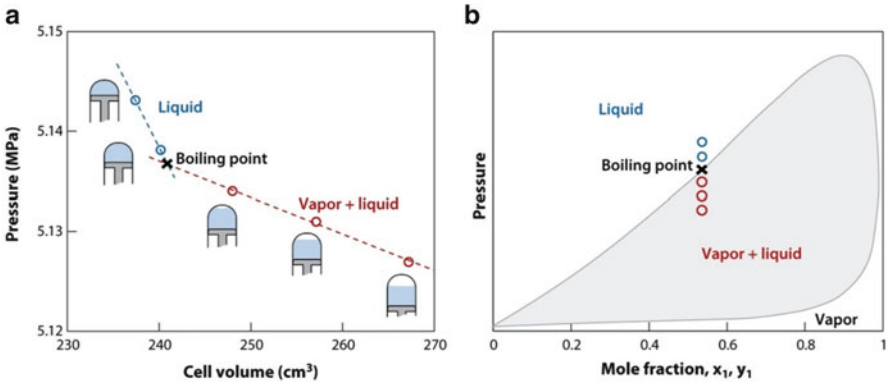

Fig. 1.8 A boiling-point determination with a synthetic-visual and a synthetic-nonvisual method (Dohrn et al. 2012). (**a**) The pressure is measured as the volume of the cell increases. The phase transition is detected visually by observing at what pressure the vapour phase appears. The phase transition is detected non-visually by observing the change in the slope of the *pV* curve. (**b**) Graphical representation of a boiling-point determination (panel a) in a pressure-composition diagram

observation. This principle is illustrated in Fig. 1.8, where the application of both visual and non-visual methods is represented.

This technique was used by Kodama et al. (2004), using an apparatus represented schematically in Fig. 1.9. This apparatus has the particularity of being one of the experimental set-ups found in the recent literature, equipped with two density meters in the recirculation loops for measuring the density of different phases. It also employs a very simple system for the variation of the volume of the cell, in which a piston is manually and directly actuated, without the use of a pressure transmitter medium. This allows the exact position of the piston and consequently the total volume of the cell to be determined with a higher precision at any instant during the experiment.

As an alternative, measurements can be performed at constant volume, for example by changing the temperature. The intersection of isochors can equally be used to determine points on coexistence curves, whenever a sharp change in the (d*p*/d*T*) slope occurs at the phase boundary. This is one of the most common methods used in the determination of hydrate formation conditions. In such experiments, only the heating process should be considered for the construction of the *pT* curves, starting from a hydrate containing system, since there is a significant sub-cooling associated with induction and growth times in hydrate formation, making the cooling *pT* curve less useful for the determinations.

Alternatively, a different technique can be applied. The temperature in the system is first lowered in order to promote the appearance of the hydrate phase, and subsequently increased in small steps. Both *pT* curves are recorded (for cooling and for heating), and the conditions at which the last hydrate crystal in the system

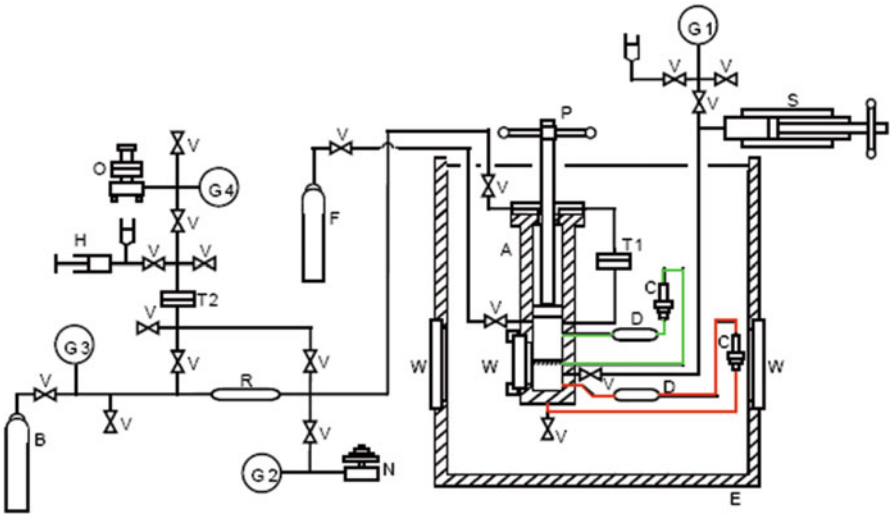

Fig. 1.9 Schematic diagram of the apparatus used by Kodama et al. (2004). The recirculation loops for the liquid and the gas phase, containing the densimeters, were highlighted for easier identification

dissociates (hydrate dissociation point) is given by the intersection of the *pT* curves from cooling and from heating (Mohammadi et al. 2008).

May et al. (2001) used a microwave re-entrant resonator in the detection of dew and bubble points in hydrocarbon systems, while Takagi et al. (2003) measured bubble point pressures using an ultrasonic speed apparatus. The excited acoustic wave used for the measurement of the speed of sound in the sample is strongly absorbed in the gas phase as compared to the absorption in the liquid phase, and so the appearance of the gas phase can be perceived by the occurrence of a change in the acoustic echo signal.

The techniques described so far, for the synthetic methods with phase change are of relatively easy application, and can prove suitable for solubility measurements of solid (or liquid) substances in a supercritical fluid for example. But other less common techniques are also available.

To measure the critical temperature of a thermally unstable substance, Nikitin and co-workers (2002) used a technique based on measuring the pressure dependence of the temperature of the attainable superheat (spontaneous boiling-up) of a liquid, using a thin wire probe to heat the sample through pulses of electric current. When the pressure in the liquid approaches the critical pressure, the temperature of the attainable superheat approaches the critical temperature. For the same type of determinations, VonNiederhausern et al. (2000) used a method in which a sample of precisely known composition is continuously displaced and heated in a capillary tube, in order to achieve very short residence times. Although resembling an analytical continuous-flow method, it is in fact a synthetic non-visual method since no analysis takes place. To determine the critical point by this method, several

temperature scans must be made in the vicinity of the critical point. Below the critical point, the temperature scan will show a flat, horizontal region indicative of isothermal boiling, while above the critical point, the transition region is no longer flat and horizontal. The critical point is inferred by the temperature and pressure where isothermal boiling is no longer observed.

Ngo et al. (2001) used a synthetic non-visual method for the determination of the solubility of solids in carbon dioxide. In the adopted procedure, the cell is initially loaded with the solid, and subsequently pressurized with carbon dioxide under permanent stirring. By periodically taking spectra (UV absorbance) of the solution, the equilibrium state is identified *in situ*. The pressure is then raised stepwise, until no further significant increase in the peak absorbance is observed, meaning that all the solids had been dissolved in the fluid phase.

Once again, similarities with other methods exist, in this case with analytical spectroscopic methods, and in fact the same apparatus could be used for both methods. Nevertheless the principles of the measurements differ greatly. In the analytical method, the quantity of solid dissolved in the carbon dioxide would be determined at each pressure from the recorded spectra, using a previously made calibration for different concentration and pressure values. In the synthetic method used by Ngo et al. (2001), the focus goes only for the pressure at which all the solid loaded into the equilibrium cell dissolves completely in the gas phase. The result relies greatly on the amount of solid inserted in the cell, but no time consuming calibrations are necessary.

Among less common methods, Oag et al. (2004) described an apparatus where the determination of phase transitions and critical points can be carried out with different methods: visually, by measuring the laser reflectance of the fluid, which is at its maximum at the critical point, and the sound velocity by using vibrating shear mode sensors, in another example of an apparatus where different methods can be used simultaneously.

Synthetic Isothermal Methods. Experiments using synthetic isothermal methods are performed without a phase transition, by measuring the pressure of a synthesized multi-phase mixture at isothermal conditions, being the phase compositions calculated through the application of a material balance. Synthetic isothermal methods are commonly used in studies of gas solubilities in non-volatile substances, such as polymers (Fonseca et al. 2012; Pfohl et al. 2002; Sato et al. 2002), oils (Bobbo et al. 2007), ionic liquids (Blanchard et al. 2001; Chen et al. 2006), or in aqueous solutions (Sidi-Boumedine et al. 2004; Dell'Era et al. 2010).

At the beginning of an experiment, the equilibrium cell is loaded with an exactly known amount of the first component, the condensed phase, and then evacuated, before setting the system to the desired temperature. The pressure in the cell should correspond to the vapour pressure of the condensed phase. Subsequently, a precisely known amount of gas is added to the cell, leading to an increase of the pressure of the system. As this component dissolves into the condensed phase, the pressure inside the equilibrium cell will decrease, eventually reaching an equilibrium value. For this reason, this method is often referred to as "pressure decay

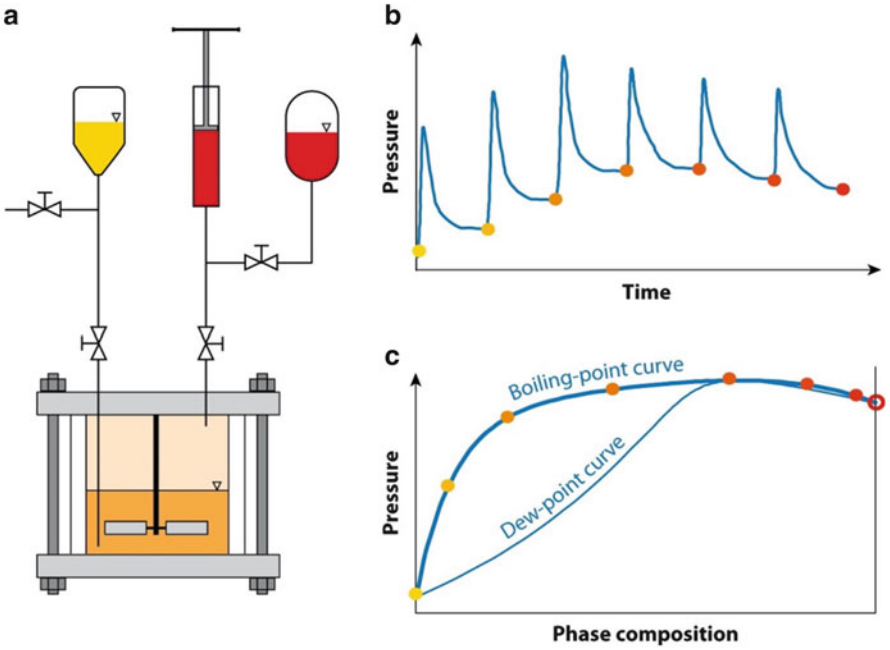

Fig. 1.10 Experimental procedure for the synthetic-isothermal method (Dohrn et al. 2012). (**a**) Experimental apparatus. (**b**) Pressure change with time during the experiment, which includes several additions of the light compound. (**c**) The resulting pressure-composition diagram

method" by some authors. A new addition of gas will lead to a new equilibrium point corresponding to a different global composition, as illustrated in Fig. 1.10.

In the ideal case, the composition of the gas phase in equilibrium can be considered as pure gas if the vapour pressure of the condensed phase is negligible, such as in solubility studies in polymers, or in ionic liquids. Based on the volume and density of the gas at equilibrium pressure (for common gases like CO_2 values are readily available from databases), the amount of gas present in the gas phase can be calculated, and the difference to the total amount of gas added to the cell, allows the calculation of the solubility.

When the vapour pressure of the condensed phase is not negligible, the gas phase composition can be estimated assuming an ideal gas mixture, or modelled using an equation of state. A common solution is to use an approximation as a starting point for the calculations. The composition of the liquid phase can then be modelled and the gas phase composition calculated from the thermodynamic model. This new gas phase composition can then be used in the calculation of a new liquid phase, in an iterative process.

Another important aspect in this method is related to the volume of the phases in equilibrium, as changes in the volume of the liquid phase occur as the gas dissolves in it. These can be negligible in cases of very low solubilities, such as of CO_2 in water, but can be significant in other cases, as in the study of the solubility of CO_2

into a polyether polycarbonate polyol (Fonseca et al. 2012) where the volume of the condensed phase increased on average 1.5 % per every 1 wt% of CO_2 dissolved in the polymer. In general, a good estimation can be achieved by considering that the volume of the condensed phase is increased by an amount equivalent to the fraction of gas dissolved at its liquid density at the temperature of the experiment. Ideally though, a view-cell is the best solution to avoid this type of errors. Additionally, view-cells present other advantages like the detection of unusual behaviours, such as foaming. It also increases the versatility of the cell, which can then also be used with a synthetic visual method (Harris et al. 2007).

A recently presented work has focused on the experimental aspects of the application of the synthetic isothermal method, giving special attention to the main sources or error and the influence of different approximations in the quality of the results (Fonseca and von Solms 2014).

Despite the fact that frequently the synthetic isothermal method rely in the use of models for the calculations of the phase compositions, they can produce results of identical quality as analytical methods, as demonstrated by Krüger et al. (2006), who compared results obtained with the synthetic isothermal method in the study of VLE for the system *n*-pentane + poly(dimethylsiloxane), with the results obtained through the gravimetric sorption method and through the use of inverse gas chromatography. These three methods differ greatly in the underlying experimental principles as well as in the complexity of the data analysis, but notwithstanding these differences, the agreement of the measured VLE data was excellent.

Synthetic Isobaric Methods. Typical isobaric experiments are performed in an ebulliometer as described previously in this chapter. In these methods the boiling temperature of a synthesised mixture is measured at isobaric conditions, and the phase compositions are calculated by means of a material balance. As opposed to analytical isobaric methods described before, no sampling or analysis is performed.

Twin ebulliometry can be used to determine the activity coefficient at infinite dilution. The temperature difference between an ebulliometer filled with the first (pure) component and a second ebulliometer (under the same pressure) filled with the first component and with a small amount of a second component (diluted solution) is measured. From the difference of the boiling temperatures, the activity coefficient at infinite dilution can be calculated.

The greatest advantage of this method is the speed of the measurements, with a pressure-temperature point typically being obtained in 1 h. But the method also presents some challenges and disadvantages such as the considerable demands on thermometry, the solubility of the buffer gas at high pressures, and thermal gradients due to pressure heads.

Other Synthetic Methods. Other methods, less common, have been described in the literature. These are sometimes specific methods use by a single researcher or research group. Information about these methods can be found in several reviews (Dohrn et al. 2010, 2012; Fonseca et al. 2011a).

1.3 Recent Trends in Measurements Comprising Food Substances

The data collected in recent reviews focusing on high-pressure phase equilibria (Dohrn et al. 2010; Fonseca et al. 2011a), denoted a clear tendency towards the increase in the application of synthetic methods. Between 2000 and 2004, synthetic methods were used in 53 % of the systems considered in the review (Dohrn et al. 2010), while for the interval between 2005 and 2008 this percentage increased to 63 %, almost two thirds of the systems reviewed. This is mainly due to the aforementioned advantages of this type of methods. Interestingly though, when only systems containing food substances are considered, the outlook is very different. An analysis of around 1,000 systems published in the literature over the last two decades showed that analytical methods are largely preferred, having been used in around 70 % of the cases. This difference from general trends denotes a certain specificity of this area of application.

A considerable fraction of the studies are done as a basis for the evaluation or optimisation of extraction processes, making the semi-flow method (analytical isothermal-isobaric) a particularly attractive option, due to the similarities with the extraction process, already mentioned. Recent examples of the application of such methods are the work of Cháfer and co-workers, who studied the solubility of solid *trans*-cinnamic acid in pure supercritical CO_2, with and without ethanol as co-solvent (Cháfer et al. 2009), or the work of Chuang et al. focusing on the solubility of β-carotene in supercritical CO_2 and propane (Chuang and Johannsen 2011).

Planeta et al. (2009) used another analytical isothermal-isobaric method, in this case open tubular capillary-column supercritical chromatography in the study of the distribution of relevant compounds between ionic liquids and supercritical CO_2.

Analytical isothermal methods are also often used, especially when the goal is to determine mutual solubilities such as in the work of Davarnejad et al. (2009) or the solubility of the gas or supercritical fluid in the condensed phase (Jenab and Temelli 2011, 2012). Knez et al. (2010) used a combination of an analytical isothermal method with a synthetic method with phase change, in the study of the solubilities of palm oil and coconut oil in SF_6.

In the small fraction of analysed works which used a synthetic method, most of the times a visual method with a variable volume equilibrium cell was used. Schwarz et al. (2011) and Schwarz and Knoetze (2012) for example, used this method in the study of the solubility of long chain carboxylic acids in supercritical propane and in supercritical CO_2. Many other examples could be given.

Other methods, like the synthetic isothermal method, are almost not used, despite being very popular in other areas of application. There are other methods with potential to be used for example in the measurement of solubilities of solid substances in supercritical fluids, but which were not found in the performed analysis of systems comprising food substances. These are for example two methods based on gravimetric determinations, but which are not gravimetric

methods. A first alternative is of very simple application, and it consists roughly in the method presented by Sherman et al. (2000). A weighed glass vial containing the solute in question is placed in the equilibrium cell, capped with a coarse filter paper. The cell is then pressurised and let to equilibrate. After this, the cell is slowly depressurised and the glassvial is removed and weighed again. The solubility can then be calculated from the mass loss.

A second and more sophisticated technique, involves a similar procedure, of detecting a mass loss, but using a quartz crystal for increased sensitivity. As mentioned previously, this method was used by Guigard et al. (2001) precisely in the determination of low solubilities in supercritical fluids. A small mass of solute is deposited on the quartz crystal and the solubility is measured by observing the change in the frequency of the crystal as the solute dissolves in the supercritical fluid. In this method the preparation of the experiment is somewhat more complex, and the apparatus involves a higher degree of electronics, when compared to the first option just presented.

As denoted at the beginning of this chapter, very often it is not possible to assess which method is the most appropriate for a specific determination, as different methods have advantages and disadvantages. Other experimental methods could therefore be suggested.

1.4 Important Experimental Aspects for the More Frequently Used Methods

Having identified the most frequently used methods in the determination of phase equilibria in systems containing food substances, it is important to focus the attention on several topics which are crucial for a high quality of the results, but that are often not published or even mentioned, namely experimental details that are usually part of the know-how developed over years of experience in the research laboratories.

Concerning the analytical methods with sampling, the most important challenge is the sampling procedure and the effects related to it, like the pressure drop during sampling from high-pressure cells. Challenges of the individual steps of the sampling procedure as well as of the effect of sampling on the remaining system were deeply analysed in a recent work (Peper and Dohrn 2012). For minimizing sampling errors, special care must be taken to ensure that the sample is withdrawn from a homogeneous, equilibrated phase, that no sample is retained in the sampling line, that a chemical change of the components during sampling due to reaction with components of the air, materials in the sampling line or due to ongoing reaction like polymerization can be excluded, and that a loss of depressurized components (especially highly volatile components) is prevented.

Sampling leads to a change of the total composition in the equilibrium cell and—if no countermeasures are taken—to a pressure drop. Both effects lead to different

compositions of the equilibrated phases as well as to a different level of the phase boundaries in the cell. These effects must be carefully considered if more than one sample is taken. Many of the solutions used to minimize or to avoid the effects of pressure drop during sampling were already mentioned earlier in this chapter. The influence of the sampling procedure in the equilibrium is affected by many aspects, e.g. by the volumes of the different phases in equilibrium.

Also used very often are the analytical isothermal-isobaric methods, and more specifically the semi-flow method. In the flow methods, the accuracy of the results depends largely on the choice of the flow rate. Low flow rates lead to long experiment times during which the conditions of the experiment, like pressure, temperature and flow rate, have to be kept constant. If the rate is too high, other errors may occur, with different consequences. A high flow rate reduces the time available for equilibration and can lead to negative errors in the measured solubility. On the other hand, entrainment is also a concern, particularly when the solute is a liquid, which would result in an excess error in the solubility. Chen et al. (2011) used a long vertical tube above the equilibrium cell as a buffer zone to avoid entrainment.

To deal with the possible problems of not achieving a true equilibrium in the experiments, Sauceau et al. (2000) used an equilibrium cell with three compartments, equivalent to three cells in series. Another possible source of problems is the eventual occurrence of partial condensation of the solute from the saturated vapour stream in the tubing, in particular inside and immediately after the expansion valve. This undesired and non-reproducible hold-up of the solute can lead to a scattering in the results in the order of 10 % (Sovova et al. 2001). To collect precipitated solute at the end of an experiment from the tubing and from the expansion valve, Takeshita and Sato (2002) used a stream of carbon dioxide after having blocked off the equilibrium cell.

Another possible problem in the study of the solubility of solids in compressed gases or in supercritical fluids is channelling, which would lead to negative errors in the measured solubilities. To prevent this, a packed bed can be used. One common solution is the use of simple glass beads, coated with the substance of interest. A distributor, which can simply be a piece of glass wool, is placed before the packed bed, assuring a uniform flow distribution. This was done by Ferri et al. (Takeshita and Sato 2002; Ferri et al. 2004) who describe an experimental technique that allows the measurement of high concentrations of dyestuff in a supercritical fluid. The authors use a second pump to stabilise the flow rate of the fluid in the extractor, damping the pulses of the first pump. Glass wool before and after the packed bed guarantees a uniform flow distribution and prevents particle entrainment. A line bypassing the extractor allows solubility measurements at high concentrations. It dilutes the saturated fluid stream with clean carbon dioxide and reduces the risk of valve clogging and flow rate instability.

In order to overcome the problems related with the depressurization process, Pauchon et al. (2004) developed a semi-flow method that works without pressure reduction. The effluent vapour-phase flows into the top part of an autoclave which is filled with mercury. The use of mercury, acting as a piston, allows obtaining a

precise adjustment of the vapour flow and avoids pressure changes that lead to solute precipitation. Sampling at isobaric conditions is performed with a six-port valve. As mentioned for other cases, special attention is required during the regeneration of mercury and cleaning of the apparatus, due to the high toxicity of this metal.

Another method commonly used in the determination of phase equilibrium in systems containing food substances is the synthetic visual method with phase change. As described before, these methods are based on the disappearance of an existing phase, or on the appearance of a new one. In the most common procedure, the initial conditions are selected in order to promote the existence of one single, homogeneous phase in the system. The pressure is then decreased until a new phase appears. Supersaturation might occur, particularly when the pressure is reduced fast, leading to a pressure of phase detection that is below the equilibrium pressure. If the procedure is reversed, starting in the two-phase region and increasing the pressure until one of the phases disappears, another challenge has to be overcome: for each new pressure a new equilibration is needed since the compositions of the phases change with pressure. Therefore, enhanced equilibration, e.g. by stirring is important. Ultimately, a combination of the two techniques can be used, providing better results and allowing the experimentalist to evaluate if the chosen pressure variation rates are adequate for the type of equilibrium in study. The existence of dead volumes such as connections to valves at the top of the cell may also lead to a wrong pressure being measured for the phase transition.

Properties which can influence the application of this method are for example viscosity and surface tension. The last will determine if the disappearing gas phase is made of a single gas bubble, or very small bubbles, sometimes barely detectable by naked eye. The results from visual phase detection can depend on the person performing the experiment and on its experimental experience. To reduce the number of error sources, videotaping the phase transitions, including information on pressure and temperature, is recommended. Non-visual methods, like the phase change detection by interpretation of the pressure vs. volume plot reduce most of the problems of visual phase detection.

Attention to these and other sources of errors are of primary importance in the application of the different methods. The frequently posed question is which method is the most appropriate for a specific determination, but the correct application of whatever method was chosen is far more important, as demonstrated by several examples in literature. One of such examples was given by Kodama et al. (2008), concerning the study of the phase equilibrium for the system CO_2 + iso-propyl acetate. The authors first performed the study using a synthetic non-visual method (change in the slope on the pressure vs. volume plot), and compared their results with values from literature. The results regarding the solubility of CO_2 were significantly higher than those previously presented by Cheng and Chen (2005) who used an analytical isothermal method with an uncertainty estimated by the authors of 0.2 mol%. Confronted with the discrepancies, the authors repeated the determinations, this time using the exact same experimental method as Cheng and Chen. The results of the new measurements differed 0.9 mol%

from those obtained first with the synthetic method, whilst the two data sets measured through the same method by different authors differed by 7.9 mol %.

In another case, two sets of data regarding the phase equilibria for the system CO_2 + vinyl acetate (Byun and Shin 2003; Stevens et al. 1997) show discrepancies of around 23 mol %, although in one of the works the estimated uncertainty of the method used was of 1 mol %. Even for systems which have been investigated several times and by different researchers, the data sometimes reveals considerable discrepancies between the results obtained by different research groups. This was observed for example by Folas et al. (2007b), who gathered literature data regarding the solubility of methane in water.

In some cases, the discrepancies between the results can be so large that the result is a completely different phase diagram, for example as in the phase equilibria of the system CO_2 + *n*-hexadecane, frequently studied as a test to validate methods and experimental procedures. For temperatures below 330 K approx., the phase diagram shows an area of liquid–liquid equilibrium (LLE) (Virnau et al. 2004; Venter et al. 2007). Figure 1.11 depicts the results available in literature for the phase equilibria of this binary system at a temperature of 313 K (Venter et al. 2007; Tanaka et al. 1993; Nieuwoudt and du Rand 2002; D'souza et al. 1988; Charoensombut-amon et al. 1986), where the mentioned LLE region is clearly identifiable. The agreement between the different sources of data for pressures up to 8 MPa is good, excluding a couple of points obtained by Venter et al. (2007). But for higher pressures, the presence of a second liquid phase seems to have gone unnoticed in the work of Charoensombut-amon et al. (1986). The erroneous data from these authors is marked by open symbols in Fig. 1.11.

From the works considered in the figure, Tanaka et al. (1993) and Nieuwoudt et al. (Nieuwoudt and du Rand 2002) used synthetic methods with variable volume cells, whilst the other authors used analytical methods. Venter et al. (2007) and D'souza et al. (1988) used view-cells, which allow the observation of the phases present in equilibrium. Charoensombut-amon et al. (1986) make no reference to a view-cell in their description of the apparatus used. This could have been one aspect contributing for the errors in the results, but other factors can be considered. The composition of the two liquid phases does not differ greatly, and consequently their properties, such as densities and refractive index, will be very similar, making difficult the phase separation and their identification. Given that a recirculation method was used, for a fast achievement of equilibrium, a mixture of the two liquid phases might have gone unnoticed. Curiously, the authors have detected the existence of two liquid phases at 308 K. Results from the experiments performed at 323 K show the same problem as the results at 313 K, i.e., no second liquid phase was detected. At 333 K, the results obtained by Charoensombut-amon et al. (1986) are in good agreement with the majority of the available literature values (Venter et al. 2007; D'souza et al. 1988; Hölscher et al. 1989). At this temperature, the results presented by King et al. (1984) are around 10 % lower than the mentioned literature sources (Venter et al. 2007; D'souza et al. 1988; Charoensombut-amon et al. 1986; Hölscher et al. 1989).

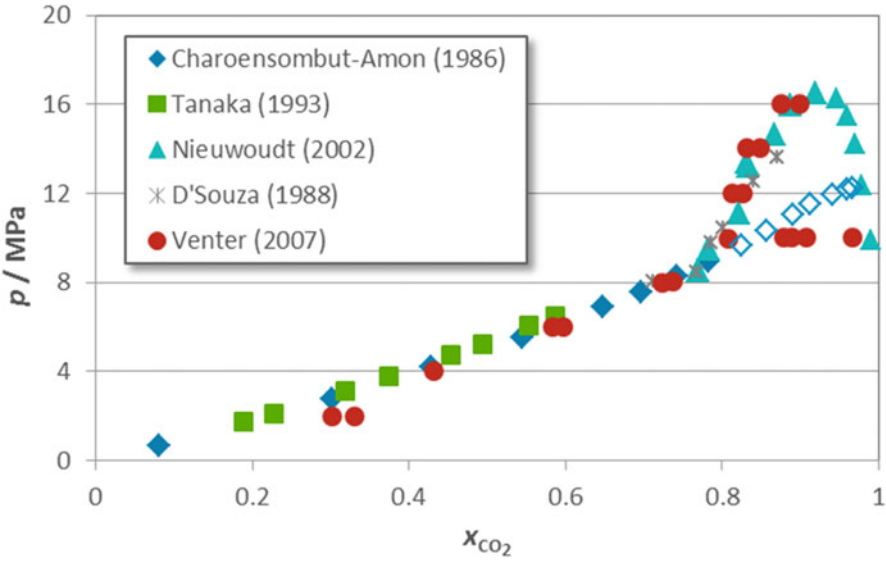

Fig. 1.11 Literature results for the phase equilibria of the system carbon dioxide + *n*-hexadecane, at 313 K (Venter et al. 2007; Tanaka et al. 1993; Nieuwoudt and du Rand 2002; D'souza et al. 1988; Charoensombut-amon et al. 1986)

All these examples demonstrate that often, it is not sufficient to know which method is the most appropriate for a specific determination. The experimentalist also plays a crucial role. Experience and know-how, are the key for high-quality results, since many experimental details are often not mentioned in publications. Simple experimental details can be very important, as recently demonstrated by Fonseca and von Solms (2014). These authors have shown that in the application of synthetic isothermal methods, the proportion between the liquid and the gas phase has a severe influence on the effect that different sources of errors can have on the quality of the results. The need for qualified laboratories with experienced staff has been acknowledged by both the academia and the industry (Richon 2009; Hendriks et al. 2010).

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Chapter 2
High Pressure Phase Equilibrium Engineering

N. Gañan, P. Hegel, S. Pereda, and E.A. Brignole

2.1 High Pressure Processing of Food Additives and Bioactive Compounds

During the last decades, a tendency to design and develop healthier and safer products and sustainable processes has grown in importance, based on "green chemistry" principles such as more efficient use of energy, replacement of traditional organic solvents by less contaminant alternatives and use of renewable raw materials. These changes are promoted by environmental concerns and governmental regulations, as well as a higher commitment from the consumers, who are gradually modifying their habits and preferences towards more "natural" products.

In this context, high pressure and supercritical fluid technologies appear as an attractive alternative to traditional processes. Most commonly used near critical and supercritical fluids (SCF) namely carbon dioxide, ethane, propane are considered "green solvents", because they are gases at ambient conditions and therefore leave no residue in the final products after depressurization. Moreover, because of their relatively low critical temperature, thermal degradation of natural products and the subsequent generation of undesirable compounds are minimized or avoided. In general, SCF are compatible with food and pharmaceutical products due to their very low toxicity; they are non-expensive and usually non-reactive.

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From a physicochemical point of view, the main advantage of supercritical solvents is that their density (and therefore all density-related properties, like solvent power) can be tuned over a broad range, from a liquid-like to a gas-like state, by simple changes in pressure and/or temperature. Also, the phase condition can be modified by properly adjusting these variables in complex systems.

In the case of natural products processing, new and promising opportunities arise from the use of supercritical fluids as solvents, anti-solvents, precipitation and separation agents or reaction media. There is an extensive literature concerning the potential applications of high pressure and supercritical technologies in the field of food industries, including the extraction, fractionation and purification of high added value ingredients, additives and bioactive substances, precipitation and encapsulation, polymer impregnation and chemical reactions.

The supercritical fluid extraction (SFE) of solid vegetable materials is currently the main application, considering the number of published research and commercial developments that are next reviewed. The decaffeination of coffee beans and the extraction of hops, spices and essential oils were the first processes successfully applied at industrial scale. Numerous plant species are proposed as raw material to obtain aromatic or bioactive extracts. In fact, plant extracts are now regarded as potential sources of valuable active compounds, such as monoterpenes, sesquiterpenes, diterpenes, flavonoids, carotenoids, phenols, etc. with antimicrobial, repellent, antioxidant, preservative and other properties (Pereira and Meireles 2010). In general, their content in the raw material is low (0.1–10 %), and the co-extraction of undesired compounds should be minimized to avoid subsequent purification steps. Therefore, research efforts are focused on improving extract yield and selectivity, by the optimization of extraction operating pressure and temperature, the use of cosolvents of different polarity, as well as increasing extraction rate, by reducing mass transfer resistances with a suitable raw material pre-treatment. The extract is generally recovered in a separation vessel by simple depressurization. If several compounds or fractions are co-extracted, they can be recovered separately using a series of separation vessels operating at different pressure and/or temperature. There are in the literature extensive reviews on SFE of aromatic and bioactive compounds from plant species (Burt 2004; Reverchon 1997a; Reverchon and De Marco 2006; Herrero et al. 2010). A list of selected cases is given in Table 2.1.

The affinity of non-polar supercritical fluids for lipid-type materials has been applied in the extraction of fixed oils, as an alternative to the traditional extraction with organic liquid solvents, like hexane. Non-flammable mixtures of propane and CO_2 have been proposed as solvents for seeds oil extraction (Hegel et al. 2007). SFE is particularly attractive when it comes to “specialty” oils, i.e. high value oils obtained from nuts (like almond, walnut, peanut, etc.), seeds (flax, grape, etc.) and cereals (rice bran, wheat germ, oat). These oils contain high concentration of bioactive lipid-soluble components, like polyunsaturated fatty acids, squalene, tocopherols, etc., as well as characteristic aromatic compounds, which are lost

Table 2.1 Typical applications of supercritical fluid extraction

Plant	Type of extract	Ref.
Aniseed	Essential oil	Rodrigues et al. (2003)
Black pepper	Essential oil	Ferreira et al. (1999), Perakis et al. (2005)
Chamomile	Sesquiterpenoid lactones	Kotnik et al. (2007)
Green tea	Catechins, polyphenols	Chang et al. (2000)
Lavender	Essential oil	Reverchon et al. (1995a)
Origanum	Essential oil, carvacrol, thymol	Simándi et al. (1999), Fornari et al. (2012)
Peppermint	Essential oil	Roy et al. (1996)
Rosemary	Essential oil	Fornari et al. (2012), Ibáñez et al. (1999)
Sage	Essential oil, diterpenes	Fornari et al. (2012), Reverchon et al. (1995b), Glisic et al. (2010)
Tagetes minuta	Essential oil	Daghero et al. (1999)
Thyme	Essential oil, thymol	Fornari et al. (2012), García-Risco et al. (2011)

during hexane evaporation in traditional extraction processes but preserved under SFE conditions (Catchpole et al. 2009; Temelli 2009).

In other cases, SFE can contribute to the revalorization of agricultural or industrial solid wastes by recovery of high added value compounds. Interesting examples are the extraction of lycopene and $\beta-$carotene from tomato skin (Sabio et al. 2003; Topal et al. 2006), the concentration of tocopherol, carotenoids and chlorophylls from residual olive husks (Gracia et al. 2011) and the extraction of diterpenes and heavier compounds with antioxidant properties from aromatic plants residues after hydrodistillation of the essential oil (Navarrete et al. 2011).

Fractionation of liquid mixtures with supercritical fluids is another promising field of application. Continuous contact columns, similar to those used in liquid extraction and distillation columns, have been proposed and developed for the concentration or purification of valuable components from natural liquid feeds (Brunner 2009). In this case, research is more oriented to the optimization of operating conditions to maximize selectivity and reduce the use of supercritical solvent, while keeping the system within the heterogeneous region. Some examples of relevance in food industry are:

- Extraction of polyunsaturated fatty acids and esters (like DHA and EPA) from fish oils (Riha and Brunner 2000; Catchpole et al. 2000)
- Extraction of squalene, carotenoids, tocopherols, phytosterols, phospholipids from vegetal edible oils (Catchpole et al. 2000; Vázquez et al. 2007; Fornari et al. 2008)
- Deterpenation of citrus peel oils (Reverchon et al. 1997; Díaz et al. 2005)
- Concentration of oxygenated monoterpenes and other bioactive components from essential oils (Köse et al. 2000; Varona et al. 2008; Gañán and Brignole 2011)

- Removal of hexane and other organic contaminants from edible oils (Espinosa et al. 2000)
- Fractionation of oleoresins (Visentín et al. 2012; Fernández-Ronco et al. 2011)

The possibility of drastically modifying solubility conditions in a supercritical fluid by changing its density has been the base for many applications in precipitation, micronization, encapsulation and impregnation processes, which have been extensively reviewed by several authors (Subra and Jestin 1999; Weidner 2009; Cocero et al. 2009).

In the rapid expansion of supercritical solutions process (RESS), the solute is dissolved at high pressure in supercritical fluid. When the solution is depressurized, the fluid solvent power decreases and the solute precipitates in fine particles. The main limitation of this process is the very low solubility of most substances of interest, requiring great amounts of supercritical solvent or costly regeneration processes. There are many examples of pharmaceutical applications, but in the case of food products research is generally focused on high added value ingredients. Caffeine (Ksibi et al. 1995), cholesterol (Satvati and Lotfollahi 2011) and phytosterols (Türk 2009) are some examples of substances micronized using this technique.

In the gas anti-solvent processes (GAS), the solute to precipitate is soluble in a conventional liquid solvent, and the non polar supercritical fluid is dissolved in the solvent-solute liquid mixture creating a high supersaturation of the solute in the liquid phase (Joye and McClements 2013). This technique overcomes solubility limitations, but additional purification steps are required for removing residual solvent.

Several food ingredients applications have been proposed, like the production of water soluble β-carotene formulations (de Paz et al. 2014), the extraction of natural antioxidants from grape residues (Marqués et al. 2013) and the micronization of oil lecithins (Magnan et al. 2000). The latter has reached industrial scale; powdered lecithin is obtained from raw-lecithin (soybean oil with 40–60 % of phospholipids) using supercritical CO_2 as anti-solvent (Weidner 2009).

The particle formation from gas saturated solution process (PGSS) is based on the ability of oils and melted fats to dissolve great amounts of supercritical fluids (like CO_2 or propane) under high pressure conditions. Gas saturated solutions are more easily sprayed, because the SCF dissolved reduces viscosity and surface tension. When the solution is sprayed to ambient pressure through a nozzle, the SCF evaporates and fine droplets of oil are formed, which are rapidly cooled by Joule-Thomson effect and precipitate. Hydrogenated oils, phospholipids, mono and diglycerides, citric acid, menthol, β-carotene, cocoa butter and natural waxes are some examples of food products powdered with PGSS (Weidner 2009).

The above mentioned techniques can also be applied to the formation of composites and encapsulation by co-precipitation of two or more substrates. There are in the literature several examples of liquid–solid composites of food ingredients, such as natural colorants (Santos and Meireles 2013) and flavoring plant extracts like rosemary and oregano (Visentin et al. 2012; Almeida et al. 2013),

among others, which are co-precipitated with powdered carriers like starch, sugars and maltodextrins.

Supercritical solvent impregnation (SSI) of polymer films with food preservative compounds has been proposed for the development of active packaging. In this case, the supercritical fluid is used not only to dissolve the active substances, but also to facilitate its diffusion into the film, which swells and plasticizes more easily by the action of high pressure fluids. After depressurization, the solutes are "trapped" inside the polymeric matrix. Impregnation of polymer films with antimicrobials cinnamaldehyde (de Souza et al. 2014) and thymol (Torres et al. 2014) are some recent examples.

High pressure and supercritical fluid technologies have been also applied as fluid media in several chemical reactions of relevance in food industry. These reactions include catalytic hydrogenation of edible oils, hydrolysis, esterification, interesterification and transesterification for the production of fatty acid esters, mono and diglycerides and shortenings. The above mentioned reactions are diffusion-controlled due to the immiscibility of the reactants. The addition of a high pressure solvent brings the system to homogenous conditions, with the subsequent increase of reaction rate and conversion (Pereda et al. 2005). Moreover, being able to control the reactants ratio also allows improving the selectivity towards the desired products. An example of this technology is the hydrogenation of sunflower oil in supercritical propane.

In the next section the typical binary phase diagrams between the SCF and families of natural products and their thermodynamic modeling will be discussed.

2.2 Thermodynamic Modeling of Solubilities in SCFs

Thermodynamic modeling of a pure solid solubility in a SCF gives a clear picture of the role of the fluid phase thermodynamic properties on the dramatic increase in solubility that is observed above the SCF critical conditions. If we apply the isofugacity criterion to the solubility of a solid in supercritical phase:

$$\hat{f}_i^S = \hat{f}_i^G \tag{2.1}$$

$$\hat{f}_i^G = \phi_i y_i P \tag{2.2}$$

$$\hat{f}_i^S = P_i^0 \phi_i^0 \exp \frac{v_S \left(P - P_i^0\right)}{RT} \tag{2.3}$$

Equations (2.1)–(2.3) show the effects of temperature, pressure and solid physical properties (vapor pressure P° and specific volume v_s) on its solubility in the SCF phase. Under pressures well below the SCF critical pressure, the solubility y_i is directly equal to the ratio of the solid sublimation pressure and the system pressure:

$$y_i = P^{\circ}/P \tag{2.4}$$

This is the ideal solubility that decreases with pressure and increases with temperature. However, when the system pressure is greater than the critical pressure of the SCF, a drastic increase in the solubility is observed due to the effect of the system pressure on the solute fugacity coefficient in the fluid phase ϕ_i.

The strong dependence of solubility with density is the basis of the Chrastil correlation (Chrastil 1982) of solute solubilities in supercritical fluids. This correlation gives the solubility (y_i) as a function of the solvent density and usually is written as follows:

$$ln(y_i) = k_i\, ln(\rho) + A_i \tag{2.5}$$

where A_i is a temperature dependent term and ρ is the SCF density under the system conditions.

The solubility of liquid solutes in supercritical fluids is also computed applying the isofugacity criterion for phase equilibrium at a given pressure and temperature:

$$f_i^L = f_i^G \quad i = 1, 2 \ldots, NC \tag{2.6}$$

The same equation of state is applied to both phases for the computation of the component fugacities. The application of cubic equations of state (EoSs) with classical mixing rules has been applied for the correlation of solutes solubilities in supercritical extraction and fractionation process when the mixtures are moderately non ideal. Recently Fornari et al. (2010) presented a review of applications of the van der Waals family of equation of state to the correlation of solubilities in SCFs. Cubic EoSs are very popular because they need in general only three component parameters (T_c, P_c and acentric factor), are able to predict or correlate vapor-liquid and liquid-liquid and vapor-liquid-liquid equilibria of subcritical and supercritical components and can give a continuous transition from heterogeneous to supercritical conditions. Improved correlation of components solubilities in supercritical fluids are obtained by fitting two interaction parameters using classical mixing rules for the equation of state covolume and attractive energy parameters. However, when there are strong polar or associating interactions, or highly asymmetric systems, the application of cubic equations with classical mixing rules is severely limited. A typical situation arises when we need to predict LL and VLL equilibria in highly asymmetric systems, like ethane or propane with triglycerides. Such capability of an EoS is highly needed in SCF applications for tuning the solvent pressure and temperature to different process needs. These systems present low energetic interaction; however, a cubic EoS is not able to correlate the multiphase equilibria with a single set of parameters (de la Fuente et al. 1997). An EoS with a repulsive term based on hard spheres like the Carnahan–Starling is more adequate for this purpose.

The group based GC-EoS model (Skjold Jorgensen 1988) is derived from two contributions to the mixture Helmholtz function (A):

$$A = A^{rep} + A^{att} \tag{2.7}$$

This model has a repulsive hard spheres term, and a group contribution van der Waals type attractive energy term. The addition of a group-based associating contribution derived from the statistical association fluid theory (SAFT) was proposed by Gros et al. (1977) to derive the GCA-EoS model.

$$A = A^{rep} + A^{att} + A^{assoc} \tag{2.8}$$

When dealing with natural products, a group contribution approach is very fruitful because a large variety of compounds can be represented with a few groups. Furthermore, the model acquires predicting capability. For example a complex molecule like linoleic acid methyl ester can be described by:

$$(CH3)_2(CH2)_{11}(CH = CH)_2(CH2COO)_1$$

The application of EoS to large molecules is hindered by the lack of information on critical properties of these compounds and most authors resort to empirical estimation methods. In the GCA-EoS model a property that is derived from critical properties or vapor pressure data, is the molecule critical diameter. Bottini et al. (1999) proposed to obtain the molecule critical diameter from experimental values of infinite dilution activity coefficients of a non polar molecule, like hexane, in a high molecular weight molecule. Later, Espinosa et al. (2002a) proposed a correlation of critical diameters as a function of the molecule van der Waals volume (r_{vdW}):

$$log(dc) = 0.4152 + 0.4128\, log(r_{vdW}) \tag{2.9}$$

In this way the critical diameters of homologous families are also obtained by a group contribution approach.

The GCA-EoS model has been applied successfully to the design of phase scenarios for the processing of food related products where critical and subcritical conditions of pure components and mixtures have been predicted in agreement with experimental results (Espinosa et al. 2002a). Case studies of phase design with applications of the GCA-EoS model are given at the end of this chapter.

The different terms of Eq. (2.8) has been the basis of a large family of SAFT-EoS where all the terms are calculated on variations and extensions of the statistical association fluid theory and results derived from statistical thermodynamics. A thorough discussion of the different SAFT models and applications has been given by Tan et al. (2008), however there have been few applications related to food products.

2.3 Phase Diagrams of Binary Systems of Solutes with SCFs

The fluid phase behavior of binary systems is useful to understand the types of phase equilibria that SCFs can exhibit with different solutes. Van Konynenburg and Scott (1980) found five types of phase behavior that cover most of experimentally studied systems. Also they have shown that all this behaviors can be qualitatively predicted by the van der Waals equation of state. Type I is typical of binary mixtures of similar size molecules and low non ideality, where complete liquid miscibility is observed up to the binary mixture critical point. Therefore, we have a continuous locus of the mixture critical points from the one of the light components to the heavy component. When the non ideality of the binary system increases we run into a region of liquid-liquid (LL) equilibria at low temperatures, but still there is a continuous locus of critical points between the two pure component critical points. When the binary system is highly asymmetric in molecular size, even with systems with moderate non ideality a different phenomena is observed. This situation is typical of binary mixtures of SCFs like ethane, propane or CO_2 with a large variety of natural products. In this type of binary mixtures, due to the appearance of liquid immiscibility near the light component critical point, there is a discontinuity in the locus of critical points of the binary mixtures with a transition from LV binary critical points, starting from the heavier component, to LL critical points that ends in a LLV line, this is Type V behavior. This situation normally arises at high molar concentrations of the lighter component and it looks like a decrease of its solvent power near its critical point, creates a segregation of a second liquid phase. The LLV line starts in the lower critical end point and ends in the upper critical end point (UCEP). From the UCEP starts a LV critical line that ends in the lighter component critical point. Luks (1986) gives a lucid description of how molecular interactions and size asymmetry determine the different types of binary phase equilibria (Fig. 2.1). In this figure univariant lines are plotted in which there is only one degree of freedom, like vapor pressure lines, LLV lines and LV and LL critical lines.

Type IV phase behavior combines both the effects of size-asymmetry and high non ideality and finally, when the low and high temperature liquid LLV line merge, the LL critical line switches to pressures above the critical point of the light component and the Type IV behavior changes to Type III. The latter has a continuous divergent locus of critical points that starts as a LV critical and ends as a LL critical line at high pressure.

In this regard, Peters and co-workers (Peters et al. 1986; Peters 1994; Peters and Gauter 1999) have done a great deal of experimental measurements to elucidate the expected phase behavior of supercritical fluids with the homologues series of many substrates like hydrocarbons (paraffinic and aromatics), alcohols, acids, among others. The evolution of the phase behavior of CO_2, ethane and propane with molecular size, for alkenes, aromatics, carboxylic acids and alcohols are shown in

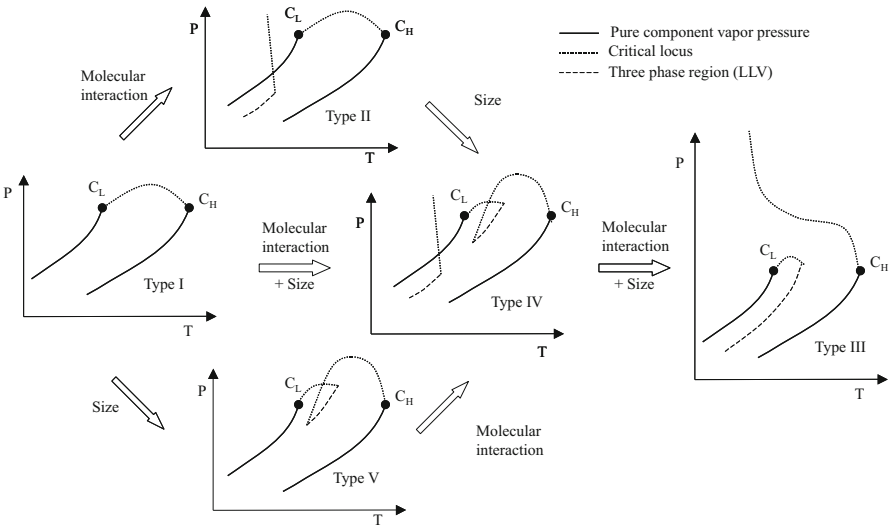


Fig. 2.1 Transition of types of phase equilibria with size asymmetry and molecular interactions

Table 2.2. Figure 2.2 shows LCEP and UCEP for propane with high molecular weight alkanes and triglycerides.

2.4 Phase Scenarios in Supercritical Fluid Processing of Natural Products

Among the supercritical solvents, low and high critical temperature (T_c) fluids depict different properties with regard to solvent power and selectivity. Low T_c solvents have critical temperatures close to ambient temperature. They have moderate solvent power and selectivities for high molecular weight or polar material. On the other hand, high T_c SCFs have high solvent power at supercritical conditions and low selectivity. CO_2, ethane and propane are typical examples of low T_c fluids and methanol, toluene and water of high T_c solvents. CO_2 and water are the most common solvent choice as a low and high temperature SCF. Both are readily available, cheap, non-flammable and non-toxic. These properties make them ideal solvents for sustainable processes. They have very different solvent properties, but we have to keep in mind that supercritical fluids can be tuned to meet the required solvent conditions. Water plays a significant role in the conversion of biomass and CO_2 in the extraction, and fractionation of high value thermally labile natural products.

Different phase scenarios are required in extraction or fractionation of natural products. As a rule in extraction of natural products from solid matrices one is looking for high solubility or complete miscibility with the supercritical fluid.

Table 2.2 Evolution of the phase behavior of the homologous series of organic compounds with supercritical fluids

Solvent	Type I	Type II	Type IV	Type V	Type III
n-alkanes					
CO_2	C_1–C_6	C_7–C_{12}	C_{13}		C_{14}–C_{32}
Ethane	C_1–C_{17}			C_{18}–C_{23}	C_{24}–C_{28}
Propane	C_1–C_{29}			C_{30}–C_{50}	C_{60}
n-alcohols					
CO_2	C_1–C_2	C_3–C_4	C_5		C_6–C_{14}
Ethane				C_1–C_{10}	C_{10}–C_{16}
Propane	C_1–C_{16}			C_{17}–C_{26}	
n-alkanoic acids					
Propane	C_1–C_{14}			C_{15}–C_{22}	
alkyl-benzene					
CO_2	C_1–C_8	C_9–C_{12}			C_{13}–C_{22}
Ethane	C_1–C_{15}			C_{16}–C_{23}	

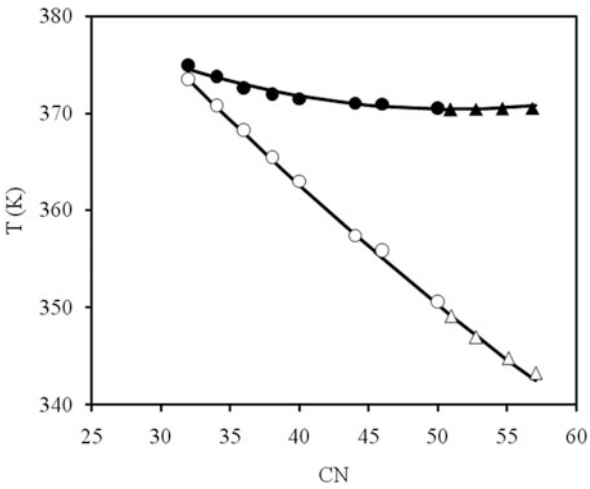


Fig. 2.2 Propane with paraffins (*circles*) and triglycerides (*triangles*). Filled and empty symbols indicate UCEP and LCEP respectively (Peters 1994)

However when the solutes of interest are extracted from a liquid phase there is a compromise between the increase in solubility of the desired components and the purity of the extracted product. In the case of fractionation, heterogeneous fluid-fluid equilibria is required to carry out the separation process. It is known that even though the potential solvents are few: CO_2, ethane or propane, their solvent properties are highly sensitive to temperature, pressure and composition (in the case of mixed-solvents). The fact that the SCFs solvent power is density dependent, and density can be changed dramatically with moderate changes in pressure or temperature, makes it possible to tune the SCF to the process needs. As an example, Fig. 2.3 shows the effect of temperature and pressure on the solubility of *Salvia*

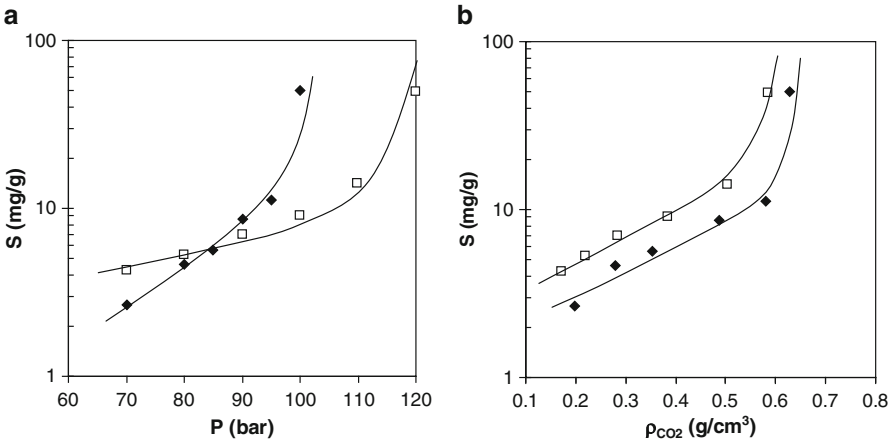


Fig. 2.3 Solubility of *S. officinalis* in CO_2: (**a**) as a function of pressure, (**b**) as a function of CO_2 density. (*filled diamond*) T = 313 K, (*square*) T = 323 K

officinalis essential oil in supercritical CO_2 (Gañán and Brignole 2011). These data when plotted against pressure show a temperature inversion point (known as crossover) that disappears when plotted against the SCF density.

The design of the phase conditions is based on the solubility and selectivity needs of the process under consideration. In extraction processes high solubility or even complete miscibility of the solutes with the SCF is required. There are two alternatives, extraction from a liquid phase or from a solid matrix (seeds or plant material). In contrast, fractionation processes requires a careful design of the phase scenario to guarantee a two phase (heterogeneous) conditions over the whole composition range of the separation process. As discussed in the previous section, these properties are dependent of the molecular size and the chemical nature of the solutes to be extracted or fractionated and their interactions with the SCF.

The phase scenarios for micronization can vary depending on the phase equilibria between the SCF and the solute to be micronized. In micronization process as rapid expansion of supercritical solutions (RESS) or gas anti-solvent, in which a cosolvent dissolves both the SCF and the solute, there are several micronization variants (Subra and Jestin 1999; Weidner 2009; Cocero et al. 2009) determined by the specific phase equilibria between the SCF, the solute and the cosolvent. Different phase scenarios can be designed to achieve the desired micronization goal.

In the case of supercritical reaction processes, by the addition of a SCF, it is possible to carry out an otherwise heterogeneous reaction under homogeneous supercritical conditions over the whole reaction pathway. This is particularly important when we are dealing with the solid catalyzed reactions of heavy substrates with gases.

2.4.1 Design of Phase Scenarios for Green Processes

The first step in the design of phase scenario is the solvent selection. For this purpose we considered mainly three solvents: CO_2, ethane and propane, because they are low temperature solvents and inherently safe to be applied to nutraceuticals, bioactive and edible products. For each solvent we will discuss the design of the phase scenario for typical supercritical separation processes. The binary phase behavior of the SCF with the main components of natural products will guide us in the selection of the conditions of pressure and temperature of operation. The evolution of the phase behavior of the homologous series of certain organic compounds with supercritical fluids is given in Table 2.2. CO_2 has been the solvent of choice, for well known reasons, in most supercritical processes. The solvent power of CO_2 is good for moderately polar and medium to low molecular weight compounds. For instance, important families in this range are the essential oils that are complex mixtures of terpenes and sesquiterpenes, oxygenated terpenic derivatives, waxes and a non volatile fraction. The extraction and fractionation of essential oils with supercritical fluids is attractive due to the use of safe solvents that leave non residues and the processes work at moderate temperatures that are suitable for thermally labile products. In Table 2.2 we can see that CO_2 with paraffinic components in the range C7–C12 have Type II phase behavior with LL immiscibility only at low temperatures. Outside this LL region there is complete miscibility and a continuous locus of the VL critical line between the critical point of the mono terpenic component and the critical point of CO_2. The selection of proper values of temperature and pressure of operation can be obtained from the phase envelope curve of a mixture of CO_2 with the main terpenic component at a given global composition in the process unit of the mixture to be extracted or fractionated.

The main coordinates in a phase envelope diagram are the mixture critical point, the temperature and pressure at the maximum pressure (crioconderbar). For example, in a mixture of CO_2 with a molar fraction of limonene of 0.02, the critical point is: $T_c = 318.1$ K and $P_c = 91.3$ bar and the $P_{max} = 147.2$ bar at $T = 376.4$ K. This is a clear indication that at temperatures lower than 370 K the operating pressure should be well below 150 bar *to avoid entering the single phase condition*. For example operating at 333 K in orange peel oil deterpenation the selected operating pressure is 100 bar (Díaz et al. 2005). In a similar fractionation process, the separation of β-ocimene (terpene) from the oxygenated fraction of *Tagetes minuta* oil at a temperature of 323 K, the operating pressure for fractionation is 90 bar (Gañán and Brignole 2011). At lower temperatures lower pressures can be selected for the oil fractionation.

When the goal of the process is the extraction and not the fractionation the critical point of the mixture gives the minimum pressure required at T_c for complete miscibility, looking at the phase envelope diagram at temperatures above T_c the pressures of complete miscibility are readily obtained. However, complete

miscibility is not always the goal; rather—a compromise between solubility of the desired component and selectivity of the process.

Table 2.2 shows that binaries of CO_2 with alkanes of carbon number higher than 14 (for instance sesquiterpenes) exhibit a Type III behavior.

In supercritical process we have to deal with size-asymmetric mixtures and, therefore, we should expect Types III, IV or V phase behavior, i.e. mixtures that show liquid phase split at temperatures near the critical point of the solvent. Figure 2.4 illustrates these types of phase diagrams. If we are looking for high solvent power we should avoid diagrams with a divergent L1 = L2 critical curve (cases "a" and "b" in Fig. 2.4) since in the near critical region, where we should operate our process, the system will show liquid immiscibility. Of course, the more favorable diagrams are of Type I or II like the ones that we have already discussed for essential oil components; a type III behavior with no rapidly divergent L1 = L2 critical curve, may be also feasible for extraction or fractionation since by increasing the pressure high enough the liquid immiscibility is avoided. An intermediate situation is that given by case "c" of Fig. 2.4—a divergent critical line with a low negative, or near null, slope in the critical region—for which homogenous liquid phase but at pressures somewhat higher than the required for case "d" (Type V) can still be achieved. An interesting example of this type of behavior is the concentration of docohexanoic acid methyl ester (DHA) and eicopentanoic acid methyl ester (EPA) by fractionation of a fish oil methyl esters mixture. The fractionation temperature has to be low (333 K) to avoid thermal degradation of the mixture. The fractionation pressure is determined by looking at the type III binary diagram of CO_2 with methyl oleate (a representative component of the lighter fraction to be removed overhead) to select a pressure in the vapor-liquid region of Fig. 2.5. At 333 K, a pressure of 145.0 bar is chosen for the fractionation process (Espinosa et al. 2002b, 2008). At a higher pressure, for instance 175 bar, the system will be in a single phase region and liquid–vapor fractionation will not be possible.

It is interesting to compare ethane with CO_2 as a SCF solvent for orange oil deterpenation and fractionation of fish oil methyl esters. Using a similar approach the operating pressures for deterpenation with ethane is 63 bar. If we compare the pressures for both solvents ethane and CO_2 in reduced coordinates they are very similar and close to $P_r = 1.5$ (Raissi et al. 2008). The reduced pressure for the concentration of DEA and EPA are again similar however the values are higher ($P_r = 2$). Higher pressures are required to increase the solubilities of heavier compounds in the supercritical phase. The concentration of $\alpha-$tocopherol from deodorizer distillate FAME follows a similar approach and a pressure of 150 bar was used by Fang et al. (2008) to remove the methyl esters and finally a pressure of 200 bar was used to recover overhead the enriched tocopherol fraction.

The solubilities of triglycerides in the carbon number range of C50–C60 in CO_2 are extremely low and the system presents a type III behavior with LL immiscibility up to very high pressures. This behavior can be advantageous to recover valuable components or eliminate contaminants from the vegetable oil by operating at pressures in the (150–200) bar range. Examples of this behavior are the removal of squalene or oleic acid from olive oil (Brignole and Pereda 2013).

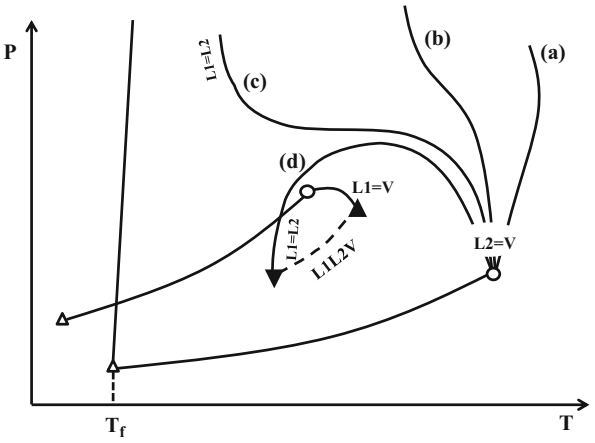


Fig. 2.4 Binary phase behavior of types III, IV and V

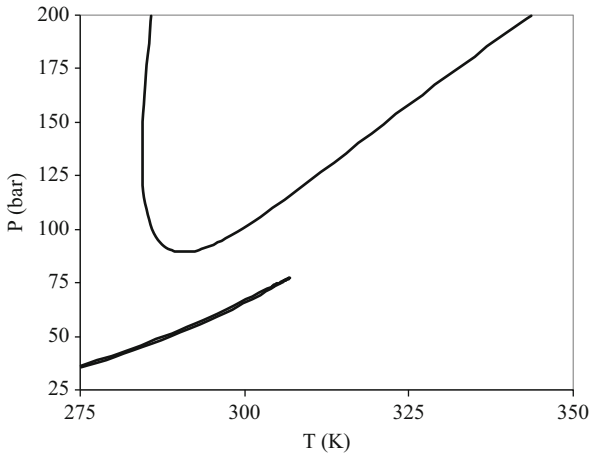


Fig. 2.5 General phase equilibrium diagram of CO_2—methyl oleate

Propane is a better SCF solvent for high molecular weight and polar material. For instance, it is completely miscible with alcohols up to C16 and with carboxylic acids up to C14. With triglycerides of carbon number less of C30 has Type I or II phase behavior and above it is Type V up to C56 (Fig. 2.2). The Type V behavior of propane was the basis of the Solexol process for oil refining using propane under selected conditions of pressure and temperature. At temperatures below the oil-propane LCEP, propane and the oil are completely miscible and only colored material and highly polar material are not dissolved. However, if the temperature is increased a liquid phase is segregated where vitamins and carboxylic acids are extracted. At higher temperatures the solubility of the oil in the propane phase is very low and propane can be used to remove contaminants or valuable products from the oil (Espinosa et al. 2000). Another interesting approach in the use of near critical fluids is the use of mixed-solvents, for instance of CO_2 and propane, to meet

requirements of non-flammability and high solvent power of SCFs solvents for fixed oils (Hegel et al. 2007). In what follows we analyze two case studies of supercritical phase design for fractionation and extraction.

2.5 Case Study: Essential Oils Fractionation

Essential oils fractionation is an interesting example of the application of a supercritical fluid as a separating agent for the concentration or purification of particular components of a complex mixture. Essential oils are natural liquid mixtures of dozens and even hundreds of terpenic compounds, including hydrocarbon monoterpenes (MT), oxygenated monoterpenes (OT) and sesquiterpenes (ST), along with small quantities of other high molecular weight compounds, like waxes, pigments and flavonoids. Some of them show a strong biocidal or repellent activity against insects, weeds, microorganisms and other common pests (Nerio 2010; Bakkali 2008; Juliani and Zygadlo 2000).

In general, the oxygenated compounds represent the most valuable fraction, because they usually carry the characteristic aromatic notes of the oils and exhibit higher bioactivity. On the other hand, hydrocarbon monoterpenes are unstable and easily oxygenated, generating undesirable off-flavors, and their bioactivity is lower. Therefore, the goal of supercritical fractionation of essential oils is the selective removal of these undesirable compounds and the concentration or purification of the oxygenated fraction.

One of the first applications of supercritical fractionation of essential oils was the deterpenation of citrus peel oils (Barth et al. 1994; Reverchon 1997b). These oils, obtained from orange, lemon, bergamot, etc. by direct cold pressing, contain large quantities of monoterpenes (namely limonene), while the valuable aromatic components (linalool, citral, decanal) represent only a minor fraction of the oil—sometimes less than 2 wt%. The partial removal of the monoterpene fraction produces "folded" oils in which the oxygenated fraction is concentrated several times, increasing its quality, stability and value. Different fractionation strategies were proposed, in order to increase process selectivity and reduce solvent consumption.

In a fractionation column, the oil flows counter currently with a stream of supercritical fluid, the different compounds distribute between the coexisting phases according to their relative volatilities. In this way, the solvent phase or "extract" is enriched in the most volatile compounds (MT), while the liquid phase or "raffinate" is enriched in less volatile and heavier OT and ST. Process selectivity is determined by the relative volatility among the components to be separated, and, therefore, it strongly depends on the pressure and temperature conditions and the composition of the system, determined by the solvent to feed ratio (S/F).

Different column configurations are possible. The supercritical solvent is fed at the bottom and flows upstream, leaving the column at the top. The oil flows downstream and leaves the column at the bottom. If the oil is fed at the top, the

column operates as a single cascade (simple countercurrent operation); if it is fed at an intermediate stage, there is an enriching and a stripping section. In this last case, the top extract is condensed and partially recycled to the column (countercurrent operation with external reflux). The use of external reflux increases product recoveries and adds an extra variable for keeping the system within the two phase conditions. Díaz et al. (2005) have shown that there is a limiting recovery of the components of interest in a simple countercurrent separation that is given by:

$$\alpha_{12} = \phi_1/(1 - \phi_2) \tag{2.10}$$

Where ϕ_1 and ϕ_2 are the recoveries of components 1 and 2 in the top and bottom products and α_{12} is their relative volatility. When greater recoveries are required the use of external reflux is needed.

2.5.1 Criteria for the Selection of Operation Conditions

The selection of conditions for the fractionation process must ensure that the system is heterogeneous (two phases) in every stage of the column. Otherwise, selectivity can be partially or totally lost, as Köse et al. (2000) have shown in a study concerning the fractionation of origanum oil with supercritical CO_2 using different pressure and temperature conditions at the top and bottom part of the column. A preliminary calculation of the phase envelope for the system prevailing composition—taking into account all feeds—is a useful tool for delimiting the range of heterogeneous and phase transition conditions.

However, the practical range of operation is limited by several other parameters. The lower temperature limit is usually the critical temperature of the solvent, while the upper limit is determined by the thermolability of the oil components. According to this, essential oils fractionation with CO_2 is generally carried out within the range (313–343) K. Above this temperature, many compounds (specially unsaturated) can be degraded, oxidized or polymerized. The pressure limits are determined by the solvent density. It is accepted as a rule of thumb that a minimum density difference of 150 kg/m^3 between the liquid and gas phase is needed in order to allow a good physical separation (Brunner 1998). Considering that essential oils density is in the range of 800–900 kg/m^3, and assuming that the solvent phase density is similar to that of the pure fluid, the solvent density should be lower than 700 kg/m^3. However, due to the high solubility of monoterpenes in supercritical solvents like CO_2, in general this kind of system becomes homogeneous at solvent densities well below this limit, and fractionation is usually operated at pressure values in the range of (80–120) bar.

Within this range, operation conditions are selected based on *selectivity* and *solubility*. An efficient process should provide the desired separation degree minimizing the solvent consumption, in order to reduce the operation and investment costs associated to a higher column height and diameter. In general higher solubility

gives smaller solvent flow rates; however if selectivity decreases in the operating range of high solubility, then external reflux may be needed to recover the separation efficiency. Separation degree is usually evaluated in terms of product concentration (or purity) and recovery in each phase, especially for the component of interest. Depending on the column configuration, the design and operation variables analyzed are: number of theoretical stages, column pressure and temperature, top separator pressure and temperature, solvent to feed ratio (S/F) and reflux ratio (RR).

2.5.2 *Experimental Measurements and Thermodynamic Modeling*

The design and optimization of a fractionation column requires an accurate knowledge of the system phase behavior. Experimental information as well as suitable thermodynamic models are required. Even though it is not common to find in the literature phase equilibrium data of complex natural mixtures and supercritical fluids, there is a good amount of binary information for many terpenes usually found in essential oils, which can be useful for preliminary estimations. However, relative volatilities based only on binary data are not always realistic, and therefore, multicomponent information should be preferred. As an example, Fig. 2.6 shows the equilibrium ratio ($K_i = y_i/x_i$) of the common terpene α-pinene in a binary mixture with CO_2 (Brunner 1998) and in the multicomponent system *Salvia officinalis* oil + CO_2 (Gañán and Brignole 2011). It can be seen that the presence of other components (namely oxygenated terpenes and sesquiterpenes) reduces its volatility and extends the two-phase region to conditions at which α-pinene is completely miscible with CO_2 in a binary mixture.

Multicomponent phase equilibrium data can be obtained using natural oils or synthetic mixtures of two or three selected components, in a high pressure equilibrium cell or using a dynamic (or saturation) method. In general, from the direct measurement of the composition of the phases, distribution coefficients and relative volatilities are calculated at different pressure, temperature and global composition conditions. Oil solubility in the solvent phase can be determined gravimetrically.

Different thermodynamic models have been used to represent the phase behavior of these systems. Several authors have correlated the experimental information using classical cubic equations of state, like SRK and PR-EoS, with good results. Other authors apply group contribution models, like the GC-EoS (Bottini et al. 1999; Espinosa et al. 2002a). The main advantage of these models is that many different and complex systems can be represented using a limited number of group parameters. In many cases, they can also predict phase behavior with great accuracy. The use of suitable models, along with robust process simulators, allows the exploration of other conditions and configurations, considerably reducing the experimental efforts.

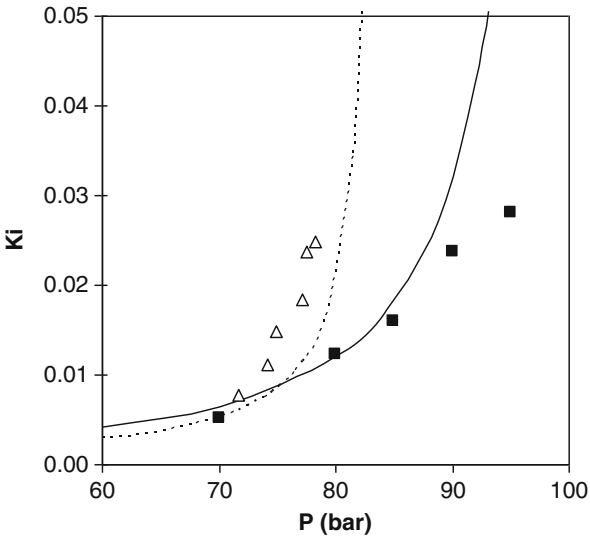


Fig. 2.6 Equilibrium ratio (K_i) of α-pinene in CO_2 at T = 313 K. Binary: (*triangle*) experimental value (Brunner 1998), Multicomponent: (*filled square*) experimental value with *Salvia officinalis* oil (Gañán and Brignole 2011), (. . .. and –) GC-EoS prediction

As an example, Fig. 2.7 shows a typical phase diagram for the system CO_2 + orange peel oil (Budich et al. 1999). Monoterpenes are highly soluble in supercritical CO_2, and therefore single phase conditions can be easily achieved at relatively low pressure levels. For instance, it can be seen that this system becomes homogeneous above 95 bar at 323 K, approximately, in agreement with data reported by other authors for continuous or semi-batch columns (Budich et al. 1999). Operation at higher pressures is possible if temperature is increased.

A general conclusion of these studies is that a trade-off between separation selectivity and oil solubility is observed. In fact, relative volatility is higher at low density conditions, increasing with temperature and decreasing with pressure. On the other hand, oil solubility in the supercritical phase increases exponentially with pressure, until reaching homogeneous conditions. Therefore, for achieving high selectivity it is necessary to operate at low density conditions, where solubility is low and higher solvent flow rates are needed. This behavior has also been observed in other cases, for example the fractionation of lavandin essential oil into its key components linalool and linalyl acetate (Sato et al. 1995), or the fractionation of *Tagetes minuta* oil. This last case will be analyzed in more detail as an example of systematic selection of operation conditions based on experimental information and supported by process simulation.

2.5.3 *Fractionation of* Tagetes minuta *Essential Oil*

Tagetes minuta essential oil has long been used as flavoring agent in a traditional way, and in the last decades numerous authors have reported biocidal activity

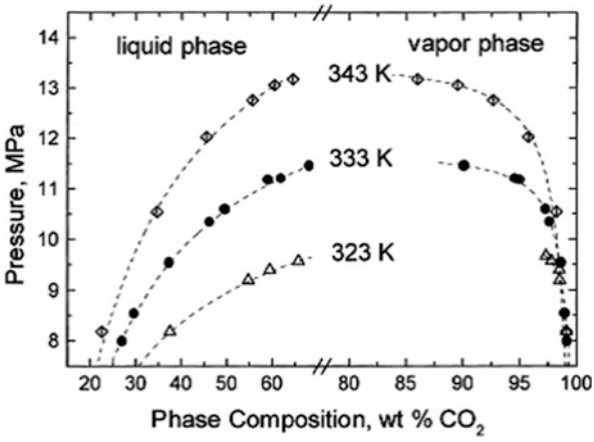


Fig. 2.7 Phase diagram for CO_2 + orange peel oil system. Experimental results (Budish et al. 1999)

against a variety of insects, nematodes weeds and other common pests of agricultural importance (Gillij et al. 2008; Scrivanti et al. 2003). A typical composition of this oil is shown in Table 2.3.

For fractionation purposes, *T. minuta* oil can be represented in a simplified way as a binary mixture of two key components to be separated: the hydrocarbon β-ocimene (MT) and the related ketone *E*-ocimenone (OT), which is the main responsible for the biocidal activity of the oil (Tomova et al. 2005). The GC-EoS model was used for representing the system phase behavior, using this simplified approach.

Fractionation experiments were carried out in a lab-scale high pressure extractor, using a dynamic or saturation method (Tomova et al. 2005). An oil sample is loaded into the column, embedded on a bed of glass particles, and a continuous stream of supercritical CO_2 is fed at given pressure and temperature conditions. The composition of the solvent phase is analyzed by GC-MS and used for estimating relative volatilities ($\alpha_{MT/OT} = K_{MT}/K_{OT}$). Oil solubility is determined gravimetrically. Figure 2.8 shows some typical results in terms of relative volatility and solubility as a function of pressure for two temperatures, along with the GC-EoS model predictions. The mentioned trade-off between both variables is clearly seen. According to these results, when temperature increases from 313 to 323 K better selectivity is achieved, and maximum operation pressure shifts from 95 to 110 bar, approximately.

In general, relative volatilities are high within this range of conditions (with values between 5 and 15), indicating that a neat separation between the key components can be achieved in a relatively small column.

GC-EoS predictions are in good agreement with the experimental information. Using this model, countercurrent fractionation was studied by rigorous simulation of a multistage column, using the program GCEXTRAC (Brignole et al. 1987). Different temperatures, pressures, solvent-to-feed ratios, number of theoretical stages and reflux ratio (RR) conditions were tested and recommended conditions

Table 2.3 Typical composition of *Tagetes minuta* oil

Component	GC area (%)
Hydrocarbon monoterpenes (MT)	
β-Pinene	1.33
d-Limonene	7.01
E-*β*-Ocimene	25.97
Other (<1 %)	0.80
Total MT	35.11
Oxygenated monoterpenes (OT)	
Dihydrotagetone	1.72
E-Tagetone	2.25
Z-Tagetone	2.33
Z-Ocimenone	11.20
E-Ocimenone	44.13
Other (<1 %)	3.27
Total OT	64.9

were selected based on separation selectivity, product recovery and solvent consumption results. From the simulation results, it is concluded that a 10-stage column is sufficient for a good separation, obtaining an almost pure OT raffinate (99.7 wt%) when operating at 323 K, 90 bar and a S/F ratio of 76 kg/kg (Table 2.4). These results are in good agreement with those proposed for citrus peel oil deterpenation, but solvent consumption is significantly lower: this can be explained by the fact that relative volatility is higher, and the amount of MT to be removed is lower than in citrus oils.

Figure 2.9 shows phase envelopes for different global compositions. It can be seen that when Solvent/Feed (S/F) ratio is increased, single phase conditions are achieved at lower pressure, for a given temperature. Selected operation conditions are also shown. These results indicate that solvent flow rate can be adjusted over a rather broad range without risk of entering the homogeneous region.

The oil composition or "quality" is an important aspect to be considered, due to the natural variability of many species associated to geographical, seasonal, crop genetic profile and environmental factors. Phase envelopes were calculated considering feeds with different MT content, for a given S/F ratio of 76 kg/kg. Single phase conditions are achieved at lower temperature and pressure values as MT content increases. For higher values (above 90 %), the system can easily reach phase transition conditions, as in the case of citrus oils. In that situation, a simultaneous optimization of all operation parameters will be needed, as reported for orange and lemon oils (Díaz et al. 2005).

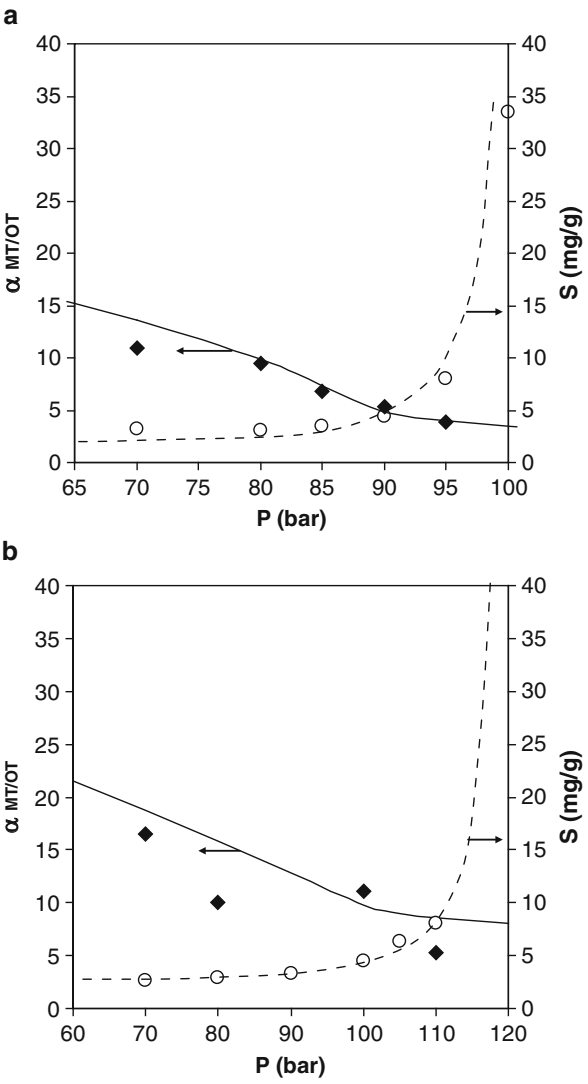


Fig. 2.8 Relative volatility and solubility for $CO_2 + T.$ *minuta* oil: (**a**) T = 313 K, (**b**) T = 323 K. (*filled diamond*) Relative volatility of MT with respect to OT, (*circle*) global solubility, (—) GC-EoS prediction

2.6 Case Study: Jojoba Oil Extraction by CO_2 + Propane Solvent Mixtures

Jojoba oil is a liquid wax extracted from the seeds of the Jojoba plant (*Simmondsia chinensis*). This wax has many functional properties that are far superior to triglycerides. Chemically, it is a mixture of high-molecular weight monounsaturated di-fatty acid esters, most of the esters are in the range of C36–C44.

As other vegetable oils, commercially jojoba oil is obtained in two stages: high quality oil is obtained by mechanical pressing of its seeds, and a secondary residual

Table 2.4 *T. minuta* oil fractionation in a countercurrent column with external reflux. Recommended operation conditions from numerical simulation (Gañan and Brignole 2013)

Variable	Value
Column temperature (K)	323
Column pressure (bar)	90
Solvent-to-feed ratio, S/F (kg/kg)	76
Number of theoretical stages	10
Oil feed stage	5
Separator temperature (K)	285
Separator pressure (bar)	35
Reflux ratio	1.0
OT concentration in raffinate (% mole, CO_2 free)	99.8
OT recovery in raffinate (%)	97.9
MT concentration in extract (% mole, CO_2 free)	96.0
MT recovery in extract (%)	98.8
CO_2 in separator vapor (% mole)	>99.9

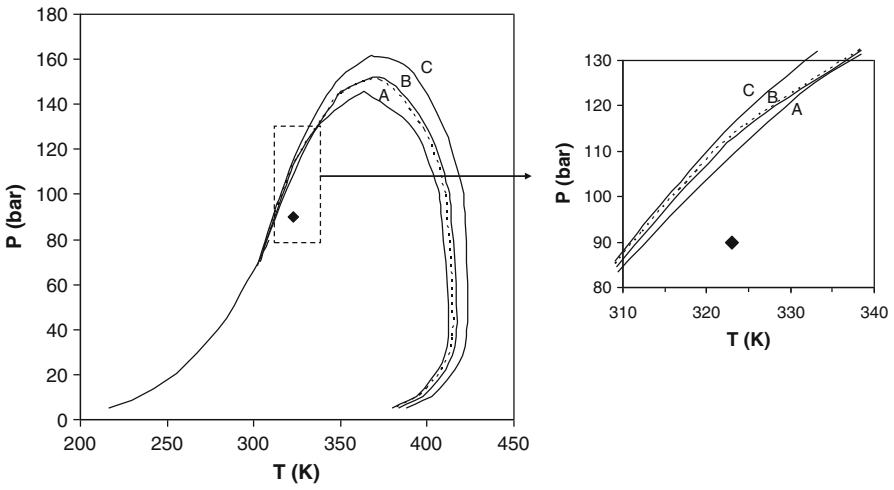


Fig. 2.9 Supercritical fractionation of *T. minuta* oil (GC-EoS prediction). Phase envelopes isopleths for countercurrent process with external reflux, n = 10; feed at stage 5. (**a**) S/F = 91 kg/kg, RR = 1. (**b**) S/F = 76 kg/kg, RR = 1. (**c**) S/F = 60 kg/kg, RR = 0.67. (- - -) Simple countercurrent, n = 5, S/F = 76 kg/kg. (*filled diamond*) Selected operation conditions

oil is recovered from the jojoba meal by percolation with hexane. However, health and environmental concerns regarding the use of solvents like hexane have placed new restrictions on the natural products industry to invest in clean technologies such as supercritical fluid extraction (Wisniak 1987).

The use of supercritical fluids for the extraction of jojoba oil has been previously studied in the literature. Stahl (Stahl et al. 1983) and Salgin (Salgın et al. 2004; Salgın 2007) studied the extraction jojoba by supercritical CO_2. However, jojoba oil

exhibits a low solubility in supercritical CO_2 even at pressures as high as 500 bar (Stahl et al. 1983). Indeed, as it is shown later, the CO_2 and jojoba oil binary system presents partial miscibility over a wide range of pressure and temperature. Salgın (2007) more recently used ethanol as a co-solvent in order to increase the CO_2 solvent power. However, the main drawbacks of these solvent mixtures are that high pressures are still required in the process to obtain good yields and most important ethanol remains in the oil after CO_2 depressurization. Therefore, the extraction of jojoba oil from its seeds using supercritical CO_2 is still not the best alternative from an economical point of view.

The used of propane + CO_2 solvent mixtures to extract vegetable oils have been considered as an attractive alternative because they have shown good results from the standpoint of solvent power, safety and selectivity (Hegel et al. 2007). It has been shown that is possible to obtain high extraction yields operating the extractor at room temperatures and pressures as low as 25 bar (Hegel et al. 2007). Mixtures of CO_2 + propane exhibit a Type I phase behavior in the classification of Van Konynenburg and Scott (1980) and a single solvent phase is obtained in a wide range of temperature and pressure. Particularly, the solvent mixture presents always a single phase (liquid or supercritical) at pressures higher than the CO_2 critical pressure (73.8 bar) (Hegel et al. 2006).

Propane has a great affinity with triglycerides and waxes, being completely miscible with vegetable oils at room temperature. It is also considered as a green solvent and it can be easily removed from the product by a simple gas depressurization. The main drawback of propane is its high flammability. However, it could be avoided by the presence of CO_2 in the solvent mixture (Hegel et al. 2006). In this case study the phase behavior of binary and ternary systems from CO_2 + propane + jojoba oil are analyzed. Then, the extraction conditions of a high pressure extractor are evaluated to obtain a good solvent power and a safe operation.

2.6.1 *Binary CO_2 + Jojoba Oil*

Figure 2.10 shows the GCA-EoS predictions of the global phase behavior of CO_2 with different wax esters (C36 + C40 + C44) according to model parameters reported previously elsewhere (Espinosa et al. 2002). The model predictions depict a Type III behavior for the binary systems, with a heterogeneous behavior over a wide range of pressure and temperature. The C36 + CO_2 system shows liquid immiscibility up to 353 K. In order to achieve homogenous conditions for an efficient oil extraction, the unit has to operate at higher temperatures and pressures above 500 bar. The shadowed region indicated in Fig. 2.10 sets the milder conditions for a feasible operation that has been reported in the literature (Salgın et al. 2004; Salgın 2007). This region includes two-phase and a single phase regions depending of the carbon number chain of the different wax esters. However in this region the solubility of the esters in CO_2 is complete or relatively high.

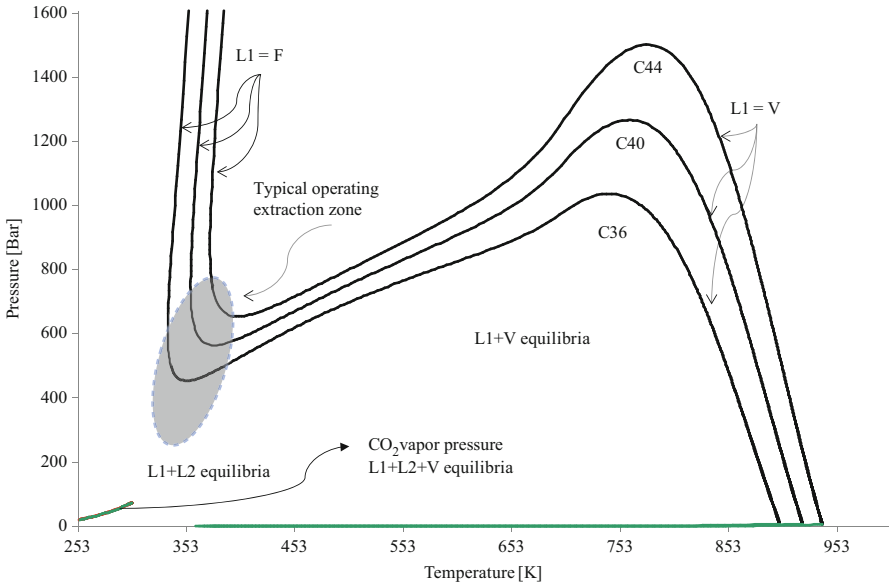


Fig. 2.10 GC-EoS predictions of the global phase behavior of wax esters (jojoba oil) and CO_2 binary mixtures. Diagram calculated with GPEC (Cismondi and Michelsen 2007)

The CO_2 + jojoba oil shows liquid-liquid–vapor equilibria at temperatures lower than the CO_2 critical temperature and pressures near the CO_2 vapor pressure (for example 298 K and 57 bar). The systems exhibit liquid–vapor equilibria at higher temperatures and medium pressures, as 373 K and 180 bar. The CO_2 + C40 binary (the major component of the jojoba oil under study) shows immiscibility of jojoba oil in liquid and supercritical CO_2 up to pressures as high as 1,000 bar, at temperatures lower than 353.2 K, the minimum temperature of the liquid-dense fluid critical curve (see L1 = F in Fig. 2.10 for C_{40} ester). However, the liquid immiscibility can be overcome at higher temperatures, for instance at 373 K and pressures higher than 565 bar.

2.6.2 Binary Propane + Jojoba Oil

Figure 2.11 shows the GCA-EoS predictions of the global phase behavior of the typical wax esters in jojoba oil and propane binary systems in a pressure-temperature diagram. The model reports a type V phase behavior with liquid-liquid equilibrium at high concentrations of propane (95 M %) in the temperature range of 350.9 K (lower critical end point) and 370 K (upper critical end point). Hegel et al. (2013) determined experimentally the phase transitions of the system propane + jojoba oil. The authors found a transition of the binary (10 wt% of jojoba oil) from liquid–vapor to liquid-liquid–vapor equilibria at 363 K and a pressure

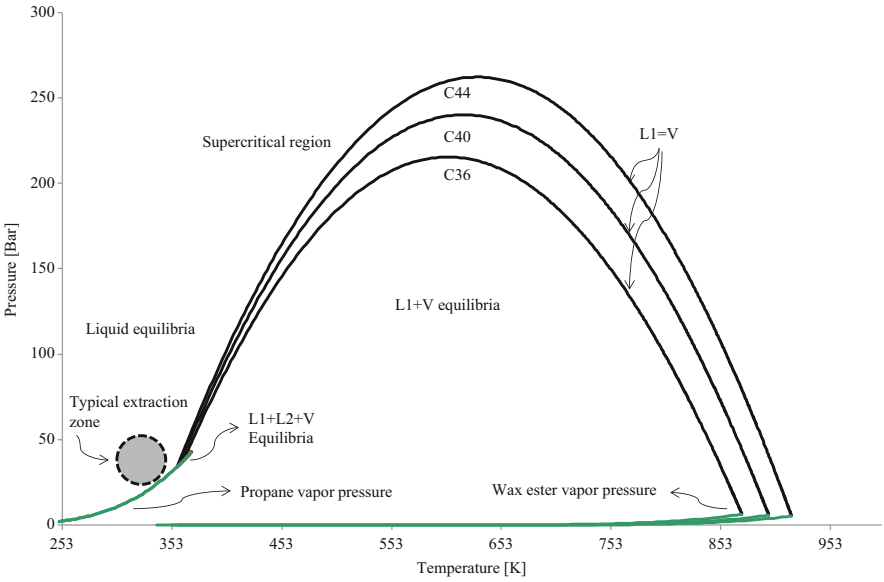


Fig. 2.11 GCA-EoS predictions of the global phase behavior of wax esters and propane binary mixtures. Diagram calculated with GPEC (Cismondi and Michelsen 2007)

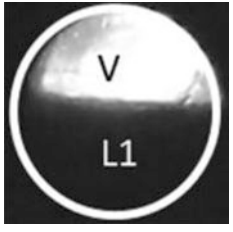
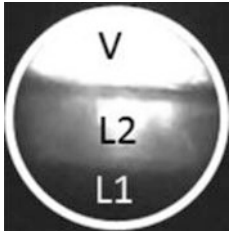
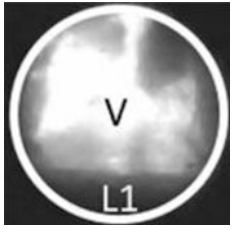
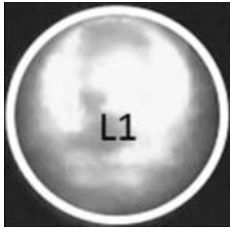
close to the propane vapor pressure. These observations are in agreement with the model predictions.

The wax esters + propane binaries show a single phase at room temperatures and pressures barely higher than the vapor pressure of the solvent. The systems also present a single phase at temperatures higher than the critical of propane and pressures higher than the critical pressure of the binaries.

2.6.3 CO_2 + Propane + Jojoba Oil Ternary System

An analysis of the binary systems is pointing out that the extractor should be operated at low temperatures (298–313 K) in the case of propane rich solvent mixtures. On the other hand, it should be operated at high temperatures (353–373 K) in the case of solvents with high CO_2 concentrations. Hegel et al. (2013) study the ternary CO_2 + propane + jojoba oil at 313 K at different pressures (Table 2.5). Particularly, the system at 40 bar shows a single liquid phase for CO_2 concentrations lower than 35 wt% and liquid–vapor equilibria is observed for solvent mixtures with greater CO_2 concentrations at this pressure. In fact, the binary CO_2 + jojoba depicts liquid–vapor equilibria at 313 K and 40 bar. It is worth mentioning that the solvent is in the limit of non-flammability under this concentration of CO_2. An increase of pressure to 60 bar shrinks the liquid–vapor region at 313 K and a solvent mixture with up to 45 wt% CO_2 still depicts complete liquid

Table 2.5 Phase transitions of the ternary system CO_2 + propane + jojoba at 313 K and different pressures (48 wt% of CO_2 in the solvent mixture, 10 wt% jojoba oil in the system). Reprinted from Hegel et al. (2013) with permision from Elsevier

Pressure			
40 bar	50 bar	60 bar	70 bar
			
Liquid–vapor	Liquid-liquid–vapor	Liquid-liquid	Liquid

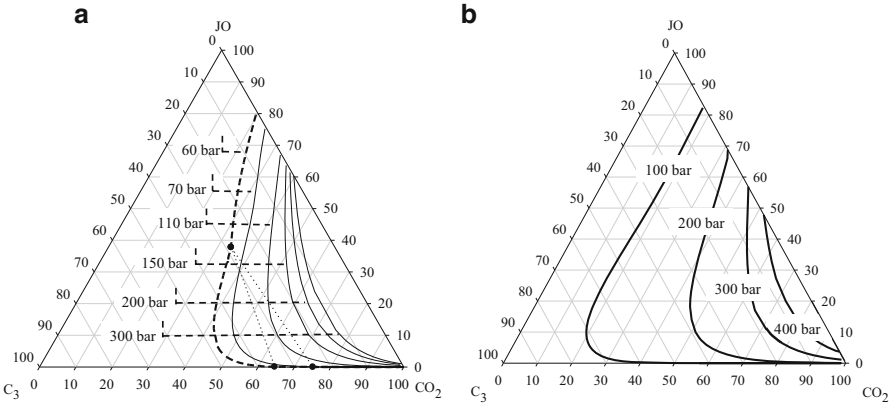


Fig. 2.12 Phase behavior of the ternary system propane (C3) + jojoba oil (JO) + CO_2 at (**a**) 313 K and (**b**) 363 K. Evaluation of the partial liquid miscibility region

miscibility with jojoba oil. However, higher CO_2 concentrations show liquid-liquid equilibria.

Figure 2.12a shows GCA-EoS predictions of the phase behavior of the ternary system at 313 K and different pressures. The binodal curve at 60 bar shows liquid-liquid equilibria for solvent mixtures with up to 65 wt% CO_2, liquid-liquid–vapor equilibria for solvents with 65–70 wt% CO_2 and liquid–vapor equilibria at higher CO_2 concentrations. A pressure increment to 150 bar produce a clear reduction of the partial miscibility region, which allows operating the extractor with solvent mixtures up to 70 wt% CO_2. Higher pressures, in the range of (200–300) bar, produce a negligible reduction of the partial liquid miscibility area. Therefore, high CO_2 concentrations in the solvent mixture (80–90 wt% CO_2) still depict liquid-liquid equilibria with jojoba oil in this pressure range.

Figure 2.12b shows the GCA-EoS predictions of the phase behavior of the solvent + jojoba oil ternary system at 363 K and different pressures. As can be seen, an increase of temperature enlarges the partial miscibility region and in consequence higher pressures are required to operate the extractor with the mixed-solvent rich in CO_2. As an example, it is necessary to increase the pressure up to 200 bar to reach a single phase system with a solvent concentration of 50 wt% CO_2. However, an increment of the system pressure up to 400 bar allows operation in a single phase region with a mixed-solvent containing 90 wt% CO_2.

2.6.4 Phase Equilibrium Engineering of the Extraction Process

There are practical considerations for the design of the operating conditions of the high pressure extractor which can be summarized as follows:

(a) In the extractor, the solvent is always in excess with respect to the jojoba oil inside the solid matrix. Therefore, the design of the extractor operating conditions should focus on the lower section of the ternary diagrams previously discussed where low concentrations of oil coexist with the solvent.
(b) In order to guarantee a good solvent power and an efficient use of the propane + CO_2 solvent mixtures partial miscibility should be avoided (Hegel et al. 2006, 2007).
(c) With regard to the safety of the entire process (high pressure extractor, separators o cyclones, recycling solvent system, pumping, etc.), the extractor should be operated with the higher CO_2 concentration for an economic process without loss of extraction efficiency.

Hegel et al. (2006, 2007) studied the operating conditions for the extraction of vegetable oil by percolation of liquid CO_2 + propane mixed-solvent. In this operation, a vapor phase remains in the extractor while the liquid percolates through the bed of grounded seeds. The extractor in this case should be designed to operate with a non-flammable vapor phase (minimum 30 wt% CO_2) and under conditions of complete liquid miscibility of the oil with the liquid solvent. The liquid-liquid–vapor equilibria must be avoided in order to attain good extraction yields. A drawback of using as much CO_2 as possible is that high CO_2 concentrations in the solvent mixture reduce the solvent mass transfer rates even in conditions of complete liquid-miscibility because of the non-ideality of the CO_2 + propane mixture.

On the other hand, the advantage of a percolation process under liquid–vapor equilibria is a low operating pressure (CO_2 + propane vapor pressure). The main drawback is that the operating region (extracting temperature and solvent composition) is highly reduced due the flammability limits and the liquid-liquid phase behavior.

In order to increase the zone of ~~a~~ feasible operation, the extractor can also be operated at pressures higher than the solvent vapor pressure, pumping a compressed liquid or a supercritical solvent through the extraction bed. This operation increases the range of temperature and solvent composition without having partial liquid miscibility problems. According to the ternary diagrams previously discussed at 70 bar and 313 K, for example, it is possible to increase the CO_2 concentration in the solvent up to 50 wt% and still obtain a single phase in the system having a good solvent power. It is possible to evaluate the plait point of each binodal curve for the ternary system CO_2 + propane + jojoba oil at different pressures to determine the maximum CO_2 concentration acceptable in the mixed-solvent. Figure 2.13 shows the result of these calculations in a diagram of CO_2 solvent concentration against pressure where the curves give the minimum pressure for a given temperature (313 and 363 K) and solvent composition that is required to get a single liquid phase in the ternary system. Shadowed regions in Fig. 2.13 indicate the feasible operating extraction conditions for both alternatives. The operation at 313 K with the solvent in liquid state (dashed line) is clearly more convenient because it allows

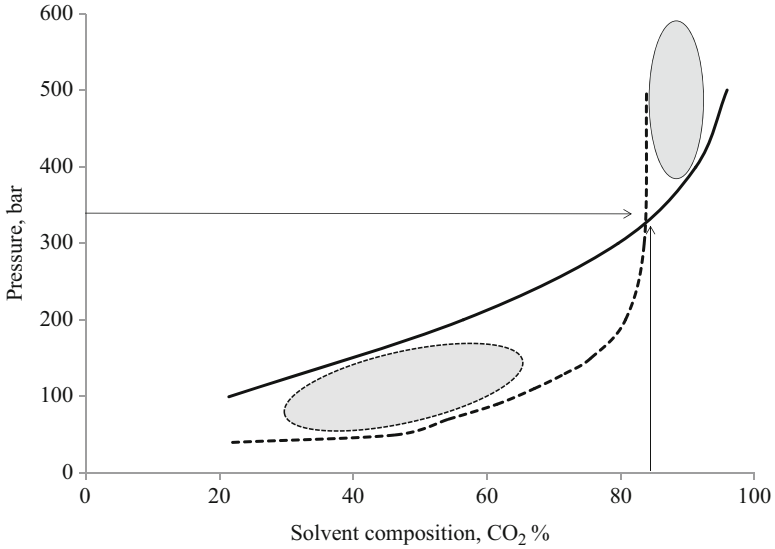


Fig. 2.13 Operating region of the high pressure extractor. GCA-EoS predictions of the boundaries of liquid-dense fluid equilibria as a function of the CO_2 solvent composition at 313 K (*dashed line*) and 363 K (*solid line*)

lower operating pressures even at high solvent CO_2 concentrations. There is also a practical limit in the solvent composition of around 60 wt% CO_2, otherwise the operating pressure should be increased to recover high solvent power.

On the other hand, the operation at 363 K results more attractive if it is desirable to use solvents with CO_2 concentrations greater than 80 wt%, with propane as co-solvent. In this case the operating pressure should be at least 350 bar to obtain a single phase in the system and good extraction yields. This pressure is acceptable for the supercritical CO_2 extraction where pressures higher than 300 bar are normally used for the extraction of vegetable oil. Salgın et al. (2004), Salgın (2007) determined a solubility of 15 g jojoba oil/kg CO_2 at 350 bar and 343 K and a solubility of 25 g jojoba oil/kg CO_2 when 8 % in volume of ethanol was added as co-solvent.

The advantage of using propane as co-solvent instead of ethanol is that it can be easily removed from the product by depressurization. Also, there would be no-solubility limitations if proper operating conditions are employed in the extraction. Figure 2.14 shows an example of jojoba oil extractions with CO_2 + propane mixed-solvent at different solvent composition (30 wt%, 50 wt% and 70 wt% CO_2) and two operating pressures (70 bar and 200 bar) (Palla et al. 2014).

The advantage of working in the single phase region in the extraction process from a solid matrix can be clearly seen from the cumulative extraction yield curves reported in Fig. 2.14. The solvent mixture with 70 wt% CO_2 exhibits a linear extraction curve at 70 bar, that is typical of extraction with solubility limitations (as it can be seen in the triangular diagram). The extraction yield for the 70 wt%

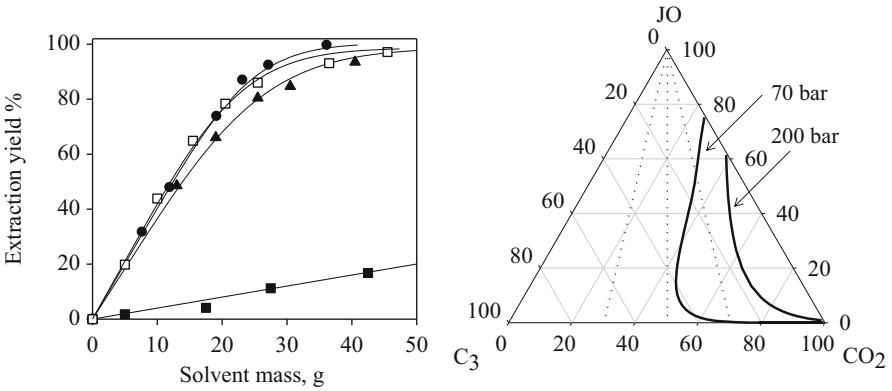


Fig. 2.14 Jojoba oil extraction by liquid CO_2 + propane mixtures at 313 K at different pressures (Palla et al. 2014). Yield of experimental extraction in *left figure*: (*bullet*) 30 wt% CO_2—70 bar, (*filled triangle*) 50 wt% CO_2—70 bar, (*filled square*) 70 wt% CO_2—70 bar, and (*square*) 70 wt% CO_2—200 bar. Triangular diagram: solid lines are the binodal of the liquid-liquid region at 70 and 200 bars and dotted lines indicate the operating lines in three mixed-solvents with different CO_2 compositions

CO_2 mixture is 15 % using 40 g of solvent, but it is 95 % for a mixture with 50 % CO_2 that has complete miscibility with the jojoba oil at 70 bar. For instance, a solvent mixture with 70 wt% CO_2 requires a higher pressure (200 bar) to avoid partial solubility limitations. Figure 2.14 also shows a triangular diagram with the liquid-liquid binodal curves at 70 bar and 200 bar together with the operating lines for the different solvent compositions. Solvent mixtures having 30 wt% and 50 wt% CO_2 were able to extract nearly the entire oil content with ca. 40 g of solvent mixture at 70 bar. Figure 2.14 shows that a higher operating pressure (200 bar) increases the feasible extraction zone and it is possible to have a high solvent power even with 70 wt% CO_2 in the solvent mixture.

Conclusions

In this chapter we wanted to highlight the importance of applying phase equilibrium engineering principles when dealing with pressure-intensified processes, which have already proved to be of great interest in the food processing industry. In order to ensure efficient operation we must be able to design and control the system phase behavior. The road to the phase design is guided by the mixture to deal with and the process goals. The mixture components' molecular interactions with the SCFs determine the binary mixtures phase behavior. The use of thermodynamic models makes it possible to explore different phase scenarios to carry out our process, identify the limits of the operating variables and select by computer simulation optimum process conditions.

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Chapter 3
Mass Transfer Models for Supercritical Fluid Extraction

Zhen Huang

3.1 General Introduction

During the last few decades, supercritical fluid extraction (SFE) has been attempted for a diverse range of practical applications because supercritical fluids possess unique physical properties intermediate between those of gases and liquids. Thus, owing to attractive gas-like transport properties and liquid-like densities, supercritical fluids have extensively been investigated for diverse porous materials fabrications (Patarin 2004; Liu and Han 2009; Tsioptsias et al. 2008; Huang et al. 2013a, b) or tremendous bioactive species extraction from various natural produces (e.g., Wagner et al. (2013), del Valle et al. (2012), Sajilata et al. (2010), Crampon et al. (2013), Kagliwal et al. (2011), Mukhopadhyay (2000), McHugh and Krukonis (1994)). In terms of mass transfer, it is known that the diffusivity of the supercritical fluid is about (10–100) times greater than that of liquid and that the viscosity of a supercritical fluid is generally comparable to that of a gas but about 100 times lower than that of a liquid (McHugh and Krukonis 1994). This means that it is much easier for supercritical fluids to penetrate porous materials with lesser resistance than for liquid solvents. In terms of the solvent strength, supercritical fluids (0.2–0.9 g/cm^3) have not much lower density than liquids (0.6–1.6 g/cm^3); hence they possess considerable solvating power (McHugh and Krukonis 1994). For this reason, extraction of targeted compounds from a large number of botanical materials by means of various SFE methods has been tremendously researched as reflected by several thousands of published scientific communications.

In view of applications to natural products, SFE is regarded as a superior extraction technique, because of its short extraction time, less organic solvent consumption, being mild to thermo-sensitive species, and able to yield clean

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bioactive extracts (Mukhopadhyay 2000; McHugh and Krukonis 1994). Among a variety of supercritical solvents considered for extraction purpose, supercritical CO_2 ($SCCO_2$) is the most widely used because it is environmentally benign, essentially nontoxic, absolutely inflammable, relatively non-corrosive even in the presence of water. Due to its low critical parameters ($T_c = 31$ °C and $P_c = 7.4$ MPa), $SCCO_2$ extraction operations can be advantageously carried out at ambient temperature, thus preventing thermal damage to labile compounds. Besides, CO_2 is relatively cheap and readily available from renewable resources in large quantities with high purity, and it is easily recoverable without harming the substrate or the extract. As thus, $SCCO_2$ extraction has become preferred as compared to traditional industrial extraction techniques, for selectively separating bioactive or thermally sensitive substances from natural plant materials (Mukhopadhyay 2000; McHugh and Krukonis 1994). More specifically, the increased consumption of vegetable extracts with food, cosmetics, and pharmaceutical applications has established the extraction of essential oils using $SCCO_2$ as an attractive alternative compared to conventional techniques, such as organic solvent extraction and steam distillation, in terms of the product quality (Temelli 2009; Herrero et al. 2010). Up to now, one can refer to a number of review articles (Temelli 2009; Herrero et al. 2006, 2010; Lang and Wai 2001; Brunner 2005; Pourmortazavi and Hajimirsadeghi 2007; Mattea et al. 2009) where recent significant progress and achievements of SFE related to plant materials have been summarized in detail. Actually, commercial applications of SFE from natural matters have been already developed in USA and Europe, including decaffeination of green or roasted coffee beans, production of hop extracts, extraction of herb and spice flavors, and extraction of oil seed lipids (Mukhopadhyay 2000; McHugh and Krukonis 1994).

Unfortunately, the laboratory research has not translated into successful commercial application as expected. Until now, there are no widely accepted designs for the purpose of SFE commercialization despite the great efforts and expenditure of resources involved to develop both technically and economically effective $SCCO_2$ extraction process. The reason is that SFE techniques for all cases used are high-pressure processes, and this limitation has subsequently restricted extraction with solid substrates to a batch wise operation (Temelli 2009; Herrero et al. 2006, 2010; Lang and Wai 2001; Brunner 2005; Pourmortazavi and Hajimirsadeghi 2007; Mattea et al. 2009). In fact, this situation has left lots of research scientists and potential entrepreneurs skeptical about the future of $SCCO_2$ extraction technology. Indeed, the use of $SCCO_2$ as an alternative tool for extraction should not be taken as a panacea, but rather must be carefully considered on a more realistic case-by-case basis. Accordingly, more rigorous and specific designs of SFE processes are required and the information on various aspects of the phenomena associated with these processes must be more accurately available. Therefore, more reliable experimental data and valid mathematical models for scaling-up purpose are in great demand.

Furthermore, in addition to the high pressure operation and the lack of enough information on scaling-up, the high investment costs for the equipment and the relatively small production capacity are the other two main reasons for the

relatively small industrial applications; obviously, these two aspects could have caused a high product price, consequently resulting in limited applications.

For the proper design of SFE processes, it is essential to have a sound knowledge of $SCCO_2$ extraction processes and appropriate mathematical representations. As addressed in recent review papers (del Valle and De La Fuente 2006; Oliveira et al. 2011; Huang et al. 2012), the whole SFE process of various solid natural matrices may be controlled either thermodynamically or dynamically, or both, in terms of solubility control, external mass transfer control or internal mass transfer limitation. These two major factors, i.e., the solubility of the extracted solute in the supercritical fluid and the rate of solute mass transfer out of the material matrices, have critically influenced the development and commercialization of the $SCCO_2$ extraction technologies. For the sake of simplicity this chapter will concentrate on mass transfer kinetics for analyte extraction from solid substrates with $SCCO_2$ only.

Up to now, a considerable number of $SCCO_2$ investigations, either experimental or theoretical, have focused on extraction of bioactive species from different natural solid matrices. Usually, the extraction is carried out semi-continuously at the designed temperatures and pressures in the vicinity of the supercritical region. The $SCCO_2$ solvent flows through a fixed bed of particles of vegetable materials in the extraction unit and dissolves the soluble substances from the plant solid bed through possible solvent–solid matrix interactions and solvent–solute interactions. The components dissolved diffuse through the pores in the matrix and finally transport to the bulk supercritical fluid. The resultant solution flowing out of the extraction unit is then released and expanded to a gaseous state, and the extracted solute becomes insoluble and precipitates in the collection unit from where the amount and composition can be determined.

In general, the extraction process can be characterized by the overall extraction curve (OEC), the plot of the cumulated extract versus the extraction time or the amount of solvent consumed during the extraction process. It is known that the extraction yield depends not only on the extraction conditions and extract solubility, but also on the physical characteristics of the extraction bed, and on the intrinsic properties of the substrates along with different pre-treatments.

In view of engineering processes and applications, mathematically simulating SFE processes in terms of time-dependent extraction curves is of great importance as the simulation results can be directly and indirectly used to develop scaling-up procedures or pilot applications based on the experimental observations from the systems studied. Moreover, an understanding of how to bridge various process variables with a theoretical kinetic model may be achieved, and the feasibility of designed extraction processes might be economically evaluated as well.

A number of kinetic mathematical models for SFE have been developed and proposed in the open literatures as discussed in details in several recent documents (del Valle and De La Fuente 2006; Oliveira et al. 2011; Huang et al. 2012). Through matching the models against experimental extraction curves, the model parameters that are physically meaningful can be determined; in turn they can not only depict the increase in extraction yield as a function of extraction time or solvent-to-feed ratio, but can also be used to predict large scale extraction curves as functions of the

process variables. From this point of view, simple empirical models (Naik et al. 1989; Nguyen et al. 1991) are of limited viability as their adjustable parameters have no physical meanings and are not adequate for any predictions, even though they sometimes could deal with SFE extraction curves very well.

On the other hand, mass transfer models which are rigorously developed from differential mass balance equations for the packed bed of solid substrates are the most valuable since they can describe all the aspects of the kinetic extraction process such as external mass transfer resistance, internal mass transfer resistance, solute–solid interactions and axial dispersion. These mathematical models contain two differential mass balances for the solute in the supercritical phase and in the solid phase, respectively, along with a solute equilibrium desorption that describes the interactions between the solute and the solid matrix. By integrating these differential equations for solid and fluid phases, time-dependent extract concentration profiles can be obtained and then the extraction yield curve can be calculated from fluid-phase concentration at the extraction unit outlet (del Valle and De La Fuente 2006).

It should be noted that when one aspect of the process prevails over the others, for example when the diffusion in the particles is much slower than the transfer of extract from the particle surface to the extraction unit outlet, the model can be simplified to describe only this controlling step. Thus, a number of mass transfer models have been proposed to characterize different mass transfer mechanisms and equilibrium relationships. Many of these mass transfer models apply the broken and intact cells (BIC) (Sovová 1994) or shrinking core (SC) (Goto et al. 1996) hypothesis, sometimes along with the heat transfer analogy concept (Reverchon et al. 1993). Application examples of the three models for SFE of various natural matters have been summarized in a recent comprehensive review (Huang et al. 2012). For example, the applications of the BIC model include the $SCCO_2$ extraction of Baizhu (Huang et al. 2011), of *Plumula nelumbinis* (Jia et al. 2009), of *Helichrysum italicum* flower (Ivanovic et al. 2011), of black pepper (Sovová et al. 1995; Ferreira et al. 1999), and of corn germ, pumpkin seed, calendula flower, and paprika fruit seed (Nagy et al. 2008). The SC model has also been used extensively for simulating SFE processes of herbaceous matrices like sesame (Döker et al. 2010), sunflower (Salgın et al. 2006), rapeseed (Núñez et al. 2011), neem (Tonthubthimthong et al. 2004; Mongkholkhajornsilp et al. 2005; Ajchariyapagorn et al. 2009), nutmeg (Machmudah et al. 2006) seeds as well as for grape and caraway seeds (Germain et al. 2005), ginger (Balachandran et al. 2006), peppermint (Goto et al. 1993), soybean meal (Kumhom et al. 2011), apricot bagasse (Döker et al. 2004), and canola seed, rosehip seed, corn germ, and olive husk (del Valle et al. 2006). The applications of the model based on the heat transfer analogy (i.e., the hot ball model) include $SCCO_2$ extraction of essential oils from basil, marjoram and rosemary leaves (Reverchon et al. 1993), from peach almond (Moura et al. 2012), and from guava leaves (Mezzomo et al. 2009), of parthenolide from feverfew flower heads (Čretnik et al. 2005), of carotenoids from microalgae (Macías-Sánchez et al. 2009), of shiitake oil from shiitake mushroom

(Kitzberger et al. 2009), of volatile oils from vetiver roots (Talansiera et al. 2008), and of oleoresin from marigold (Campos et al. 2005).

The SC model (Goto et al. 1996), however, is limited by the fact that it requires five adjustable parameters: fluid film mass transfer coefficient, desorption rate constant, adsorption equilibrium constant, axial diffusivity, and the effective diffusivity in the porous solid. Of these parameters, at least two are regressed from the experimental results while the rest are obtained from the available correlations. Thus, the accuracy of this model is ridiculously dependent on the correlations selected.

The BIC model proposed by Sovová is a first attempt to introduce a physical description of vegetable substrate. Indeed, Sovová (Sovová 1994; Sovová et al. 1995) and Sovová et al. (Sovová et al. 1994; Štástová et al. 1996) have taken into account the solid phase as divided between broken and intact cells containing the vegetable oil. The major drawback of the model is that it also requires a lot of adjustable parameters (for example, four parameters in the case of sea buckthorn and three in the case of grape seed). Hence, at least three independent data sets (three different CO_2 flow rates with all the other process parameters set at a fixed value), are required for proper modeling to obtain the best set of parameter values that validates the model. Besides, only the simplified forms of this BIC model have been tested. The merits of this model are that it uses a realistic description of the vegetable structure, and that the vegetable microstructure hypothesis has actually been verified by scanning electron microscope (SEM) analysis of almond and fennel particles (Marrone et al. 1998; Reverchon et al. 1999; Reverchon and Marrone 2001). Based on the SEM analysis results, Reverchon and Marrone (Reverchon and Marrone 2001) have modified the BIC model by retaining the internal mass transfer coefficient as the only one adjustable parameter in the model since all the other parameters were calculated from data and from vegetable microstructure. Based on this, several general aspects can be considered in the modified models of SFE of various natural products: the structure of the botanical material, the location of extracted substances such as essential oil, the adsorption on plant matrix and other forms of solute–matrix interactions, the breakage of certain cell structures, and the shape of the particles. However, the limitation of this modification is that the cell structure data required for the solution of the model are obtained by SEM images and require expertise to avoid any confusion between the oil bearing cells and biological cells like starch bearing cells. This model does not produce accurate results for seeds with very low initial oil contents. SEM analysis of the microstructure shows that specialized oil–bearing structure for seeds with very low initial oil content cannot be developed. Taking into consideration the large quantity of possible structures for botanical materials, it is obvious that mathematical modeling is still quite far from being complete.

Till present, it is still not clear whether it is appropriate to apply a particular mass transfer model in a particular situation; yet, it is meaningful to summarize the progress in mathematical modeling of supercritical fluid extraction of natural materials. Hence, this chapter reviews those theoretical models that are developed from differential mass balance equations and are extensively applied for simulating

the kinetics of SFE processes. Besides, models with analytical solution are addressed as they largely simplify the estimation of model parameters against the experimental extraction results.

In what follows, the mathematical models considered here include: (1) the most successful broken and intact cell model, (2) the shrinking core model, (3) the microstructured mathematical model, (4) the heat analogue diffusion model and other relatively simple models.

3.2 The Broken and Intact Cell (BIC) Model

3.2.1 Original BIC Model

Sovová (Sovová 1994; Sovová et al. 1995) and Sovová et al. (Sovová et al. 1994; Štástová et al. 1996) proposed the conception of the broken and intact cells and considered the solid phase as divided between broken and intact cells. The authors have distinguished the total solute available within the plant cell in easily accessible solute and solute present in the intact cell. This assumption could be reasonably true for most $SCCO_2$ extraction processes since natural materials are usually pretreated (e.g. grinding, milling or crushing) before loaded into the extraction bed. Sovová developed the broken and intact cells (BIC) model based on Lack's plug flow model in 1994 (Sovová 1994); since then many researchers have successfully applied it for modeling $SCCO_2$ extraction processes in terms of overall extraction curves, as reflected by the very large number of communications published, particularly for the case of oil rich plant materials.

Generally speaking, the BIC model is applicable when some of the extractable material is at the outer surfaces of the grounded particles or in ruptured plant cells (a result of mechanical pretreatment), and thus can be readily accessible to the SFE solvent; the rest of the material is either still in the botanical pore structure or in intact plant cells and is, hence, less accessible. Consequently, the extraction of the easily accessible solute is fast while the extraction of the less accessible solute from intact cells is much slower due to high mass transfer resistance.

According to the BIC model, the overall extraction curves can be described by a three-step procedure: constant extraction rate (CER), falling extraction rate (FER) and diffusion-controlled (DC) periods, respectively. The first linear portion is the CER period and is characterized by the convective mass transfer between the solid material surface and the fluid phase. Supercritical solvent carries the easily accessible solute from the broken plant cells during this phase. The last part of the extraction curve represents a DC period where the diffusion in the solid controls the mass transfer. During that period, the external solute in the broken cells disappears and only the less accessible solute in the intact cells is slowly extracted. The intermediate part is modeled as an FER period where both convective mass transfer and diffusion in the solid are considered. Note that in the transition phase, the solute

in intact cells starts to be extracted and the readily accessible solute continues to be extracted whereas the extraction rate drops rapidly during this period.

The details of the BIC model are given in Table 3.1. In Table 3.1 e is time-dependent extraction yield (g solute/g solute-free feed); ρ_s and ρ_f are the density of solid substrate particle (g/m^3) and that of the $SCCO_2$ fluid (g/m^3); m_{CO2} is the mass (g) of $SCCO_2$ solvent consumed and m_{bed} is the mass (g) of solute-free feed loaded in the bed; q and $\dot{q}$ are the specific mass (g solvent/g solute-free feed) and specific mass flow rate (g solvent/g solute-free feed in unit time) of solvent passed through the extraction, respectively; x_0 is the total solute concentration in the initial matrix sample and is readily estimated from the exhaustive extraction yield (g solute/g solute-free feed); x_u is the solute concentration in the untreated solid, equal to the initial concentration of the difficultly accessible solute in the intact solid ($x_{\text{i},0}$, g solute/g solute-free feed); q_{CER} is the value of q when the extraction of the less accessible solute from the intact cell begins; q_{FER} is the q value when the easily accessible solute is all extracted; y_{s} is the equilibrium solubility of the solute in the $SCCO_2$ solvent (g solute/g solvent); Z_w is the dimensionless axial coordinate between fast and slow extraction. W and Z are the dimensionless mass transfer parameter in the solid phase and in the fluid phase, respectively; k_f and k_s are the solvent-phase mass transfer coefficient (m/s), and the solid-phase mass transfer coefficient (m/s), respectively; a_0 is specific surface area of an equivalent spherical particle ($a_0 = 6(1-\varepsilon)/d_p$, m^{-1}), and $k_f a_0$ and $k_s a_0$ are the volumetric solvent phase mass transfer coefficient (s^{-1}) and volumetric solid phase mass transfer coefficient (s^{-1}), respectively.

The SFE process considered for the BIC model can be described as a $SCCO_2$ solvent flowing axially with a constant superficial velocity (u) through a fixed bed of pretreated solid matrices in a cylindrical extraction unit. The model assumes spherical geometry of pretreated solid particles (diameter d_{p}), plug flow pattern of $SCCO_2$ in the packed bed (height, H), constant bed void fraction (bed porosity, ε) during extraction, constant physical properties of the $SCCO_2$ and substrate, negligible pressure drops and temperature gradients in the bed, negligible solute accumulation and no axial dispersion of solute in the supercritical phase. As a result of these assumptions, the solute mass fraction in $SCCO_2$ (y, i.e., the solute concentration in the $SCCO_2$ fluid phase, g solute/g solvent) and the solute concentration in the solid phase (x, g solute/g solute free solid bed) depend only on the axial position along the bed (z) and extraction time (t). Readily, differential mass balance equations in the solid particles and $SCCO_2$ phase can be written as Eqs. (3.1) to (3.4), respectively (refer to Table 3.1). Along with initial and boundary conditions (Eq. (3.5)), the differential mass balance equations can be explicitly integrated, subsequently resulting in an analytical solution for the over extraction curve that is dependent on the amount of $SCCO_2$ consumed. It is unsurprising that the solution of the BIC model is a three-phase function (refer to Eqs. (3.6) and (3.7) in Table 3.1), which is respectively correspondent to the three-stage extraction procedure as reflected by the overall extraction curve.

Table 3.1 Detailed equations for the BIC model

Equation	No.
Differential mass transfer in the solid phase:	
$\rho_s(1-\varepsilon)\frac{\partial x}{\partial t} = -f(x,y)$	(3.1)
Differential mass transfer in the bulk fluid phase	
$\rho_f u\frac{\partial y}{\partial z} + \rho_f \varepsilon\frac{\partial y}{\partial t} = f(x,y)$	(3.2)
Interfacial mass transfer rate	
$f(x > x_u, y) = k_f a_0 \rho_f (y_s - y)$	(3.3)
$f(x \le x_u, y) = k_s a_0 \rho_s x$	(3.4)
Initial and boundary conditions	
$\begin{cases} x(z, t=0) = x_0 \\ y(z=0, t) = 0 \\ y(z>0, t) = y_s \\ \left.\frac{\partial y}{\partial z}\right\|_{z=H,t} = 0 \end{cases}$	(3.5)
Analytical solution	
$e = q \times y_s \times [1 - \exp(-Z)]$ for $q < q_{CER}$	(3.6a)
$e = y_s \times [q - q_{CER} \times \exp(Z_w - Z)]$ for $q_{CER} \le q < q_{FER}$	(3.6b)
$e = x_0 - \frac{y_s}{W}\ln\left\{1 + \left[\exp\left(W + \frac{x_0}{y_s}\right) - 1\right] \times \exp\left[W \times (q_{CER} - q) \times \frac{x_u}{x_0}\right]\right\}$ for $q \ge q_{FER}$	(3.6c)
$q = m_{co_2}/m_{bed}$ and $\dot{q} = q/t$	(3.7a)
$q_{CER} = (x_0 - x_u)/(y_s \cdot Z)$	(3.7b)
$q_{FER} = q_{CER} + \frac{1}{W} \cdot \ln\left(\frac{x_u + (x_0 - x_u)\cdot\exp(W\cdot x_0/y_s)}{x_0}\right)$	(3.7c)
$Z = k_f a_0 \rho_f / [\dot{q}(1-\varepsilon)\rho_s]$	(3.7d)
$W = k_s a_0 / [\dot{q}(1-\varepsilon)]$	(3.7e)
$Z_w = \frac{Z\cdot y_s}{W\cdot x_0} \cdot \ln\left(\frac{x_0\cdot\exp[W\cdot(q-q_{CER})] - x_u}{x_0 - x_u}\right)$	(3.7f)

$$t_{CER} = (x_0 - x_u)/(y_s \cdot Z \cdot \dot{q}) \tag{3.7g}$$

$$t_{FER} = t_{CER} + \frac{1}{W \cdot \dot{q}} \cdot \ln\left(\frac{x_u + (x_0 - x_u)\cdot\exp(W\cdot x_0/y_s)}{x_0}\right) \tag{3.7h}$$

Instead, the overall extraction curve can also be expressed in terms of real time used, and then two terms of t_{CER} and t_{FER}, i.e., constant extraction rate (CER) and falling extraction rate (FER) periods, may be used. According to the BIC model, t_{CER} can be defined as the time experienced till the solute extraction from intact cells begins (s) and t_{FER} as the time at which the solute extraction from ruptured cells finishes (s). They can be readily obtained from Eq. (3.7). Figure 3.1 shows one example for the constant extraction rate t_{CER} calculated for the case of $SCCO_2$ extraction of oil from potato chips (Wagner et al. 2013). As seen from Fig. 3.1, t_{CER} tends to decrease at higher pressure or higher temperatures, probably due to the increase of the driving force and then the decrease of mass transfer resistance with increased pressure or temperature.

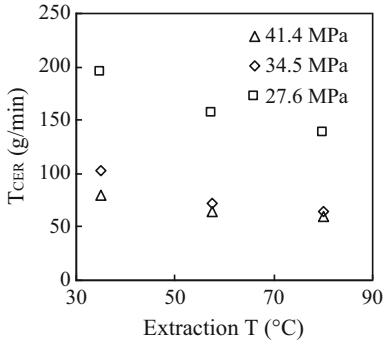

Fig. 3.1 Pressure and temperature dependence of the constant extraction rate t_{CER} for the case of $SCCO_2$ extraction of oil from potato chips (data from (Wagner et al. 2013))

Since the BIC model offers analytical solution of the equations for the cases of stationary process and low solubility of the solute in supercritical fluid, thus it is generally applicable to SFE from any kind of herbaceous materials such as leaf, flower, root or fruit and is suitable to describe the SFE of any kind of solute like essential oil, fatty oil or waxes (Huang et al. 2012; Wagner et al. 2013; Özkal et al. 2005; García-Risco et al. 2011; Bensebia et al. 2009; Kiriamiti et al. 2002; Silva et al. 2009; Rezaei and Temelli 2000; Rochová et al. 2008; Andrade et al. 2012; Moura et al. 2012; Ciftci et al. 2012; Aguiar et al. 2012; Jokić et al. 2012). As shown in Table 3.1, the BIC model includes five adjustable parameters, i.e., x_0, x_i, y_s, k_f and k_s, for data correlations although it has an analytical solution as given in Eqs. (3.6)–(3.7). Obviously, the requirement of determining so many parameters will make it difficult to use the model for regression against the experimental data. For instances, Franca and Meireles (de Franca and Meireles 2000) and Povh et al. (Povh et al. 2001) have attempted a spline fitting method for obtaining the model parameters. Ferreira and Meireles (Ferreira and Meireles 2002) have related the mass transfer coefficient to t_{CER} for the model parameter evaluations. Martínez and Martínez (Martínez and Martínez 2008) have turned to a global optimization method to estimate the parameters for predicting SFE with the BIC model. The number of adjustable parameters may be reduced from five to three by some addition information. In fact, x_0 and y_s may be determined experimentally. The solute equilibrium solubility y_s (Özkal et al. 2005; García-Risco et al. 2011) can be directly measured in $SCCO_2$ under low flow rates or conveniently evaluated from the slope of the linear part of the extraction yield curve. Based on the y_s obtained, Özkal et al. (Özkal et al. 2005) have successfully performed mass transfer modeling of apricot kernel oil extraction with $SCCO_2$ applying four adjustable parameters. They have found that the volumetric mass transfer coefficient in the fluid phase, $k_f a_0$ (varying from 0.7 to 3.7 min^{-1}), is three to four orders of magnitude greater than the volume mass transfer coefficient in the solid phase $k_s a_0$ (spanning between 0.00009 and 0.0005 min^{-1}). Furthermore, $k_f a_0$ is observed to increase with decrease in particle size (mean particle diameter ranging from $<$0.425 to 1.5 mm) and pressure (30.0–60.0 MPa), and with increase in solvent flow

rate (1–5 g/min), temperature (40–70 °C) and co-solvent ethanol concentration (up to 3.0 wt%).

The parameter x_0 is commonly estimated from the exhaustive extraction yield, taking it to be the maximum yield over all conditions investigated (Huang et al. 2011; García-Risco et al. 2011). With the known values for x_0 and y_s, García-Risco et al. (García-Risco et al. 2011) have successfully simulated rosemary $SCCO_2$ extraction carried out in a pilot-scale plant of 2 L capacity and processing 0.6 kg of grinded rosemary leaves. The average deviation between measured and calculated yields is satisfactorily lower than 2 %. Their results show that the $k_f a_0$ value regressed for the pilot-scale extraction is quite in accordance with the values for the low-scale case reported by Bensebia et al. (Bensebia et al. 2009), showing an increase with a solvent velocity increase. But the $k_s a_0$ value obtained is around one order of magnitude lower than that reported by Bensebia et al. (Bensebia et al. 2009), due to the larger particle size (0.5–1.0 mm) employed. For this reason, large amounts of solute remains inside the cell walls and the overall extraction is mainly governed by mass transfer diffusion in the solid phase, along with shorter constant extraction rate period and falling extraction rate period.

On the other hand, x_0 can be experimentally determined with organic solvent extraction (Kiriamiti et al. 2002). Kiriamiti et al. have adopted a Soxhlet extraction by hexane to measure the total oil content of sunflower seeds resulting in a value of 42.55 mass %. With this value of x_0, the oil extraction yield curve has satisfactorily been simulated with higher than 85 % accuracy. Interestingly, Silva et al. (Silva et al. 2009) have examined the influences of different x_0 values on the BIC modeling of $SCCO_2$ extraction of carqueja oil. These values include the highest yield obtained by organic solvent extraction, the extraction yield obtained over all SFE conditions and the highest yield obtained under each specific SFE condition. Their results show that the x_0 value for a specific pressure and temperature condition in SFE can be better for modeling the OEC curves. If the highest value of x_0 is used instead, the modeling turns to overestimate the yield results as a higher value means a higher amount of extractable solute. As such, the authors suggest that it may be important to define the amount of solute present in the solid materials in order to allow for a better SFE modeling.

The BIC model can simulate the overall extraction curve with the three adjustable parameters x_u, k_f and k_s, or with their equivalent terms as discussed below.

The parameter x_u, the solute concentration in intact cells, can be replaced by using a term of x_b (i.e., the initial concentration of the easy accessible solute in broken cells, $x_0 = x_u + x_{b,0} = x_{i,0} + x_{b,0}$) or r (i.e., the grinding efficiency). Although x_u or r is commonly fitted from the experimental data, one may note that x_u or r in the modeling should be taken as a constant at given conditions because x_u (or r) is only related to the particle size of the pretreated solid particles. Figure 3.2 shows the scheme and one example for the particle size effect on the parameter x_u (r). As shown in Fig. 3.2a, the parameter t_h, corresponding to the particle shell thickness where the solute of easy access is located, remains constant but the diameter of the intact particle core (i.e., d_p–t_h) varies with the particle size. Thus, when the particle size varies, the initial difficult–to–access solute content, x_u, can change

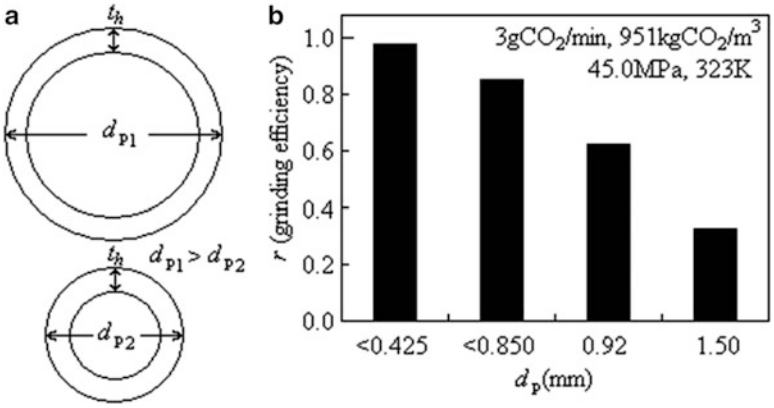

Fig. 3.2 Scheme representing the particle size effect on the parameter x_b ($d_{p1} > d_{p2}$) and one example for the r dependence on the particle size of apricot kernel samples (data from Özkal et al. (2005))

considerably as clearly shown in Fig. 3.2b for apricot kernel oil extraction with $SCCO_2$ (Özkal et al. 2005). It can be seen that as d_p, the particle size of apricot kernel samples, decreases from 1.50 to <0.85 mm, the grinding efficiency regressed using the BIC model increases significantly from 0.322 to 0.854. For this reason, the extraction rate will decrease with larger particle size and both mass transfer parameters decrease with the increase of the particle size of apricot kernel due to the larger diffusion path. As d_p decreases from 1.50 to <0.85 mm, $k_f a_0$ varies from 0.947 to 1.895 min^{-1} and $k_s a_0$ increases from 0.00019 to 0.00028 min^{-1} when extraction has been performed under $P = 45.0$ MPa, $T = 50$ °C and $\rho_f = 951$ kg/m^3 at a $SCCO_2$ flow rate of 3 g/min.

With regard to k_f and k_s, they both can be directly regressed using the BIC model from the data fitting (Huang et al. 2011; Jia et al. 2009; Ivanovic et al. 2011; Sovová et al. 1995; Ferreira et al. 1999; Nagy et al. 2008) provided the specific interfacial area a_0 is available. Based on the results obtained by using the BIC model, one can readily notice that k_f is generally 2–4 orders of magnitude larger than k_s, indicating the possible presence of both the easily accessible solute and the less accessible one. On the other hand, both $k_f a_0$ and $k_s a_0$ parameters may be preferably used instead, to avoid the effect of different ways to estimate a_0. A summary of $k_f a_0$ and $k_s a_0$ values calculated by using the BIC model for various vegetable matters has been presented in our earlier work (Huang et al. 2012). Unsurprisingly, the $k_f a_0$ values are generally 2–3 orders of magnitude higher than $k_s a_0$ since $k_f a_0$ depends on the diffusion of the oil released on the ruptured particle surface whereas $k_s a_0$ relies on the diffusion of the unreleased oil in the intact particles. The dependences of $k_f a_0$ and $k_s a_0$ on a number of process parameters have been systematically investigated in (Özkal et al. 2005), where mean particle size, solvent flow rate, pressure, temperature and modifier concentration have been examined. Both $k_f a_0$ and $k_s a_0$ are observed to increase with CO_2 flow rate, modifier content and extraction temperature, due to the

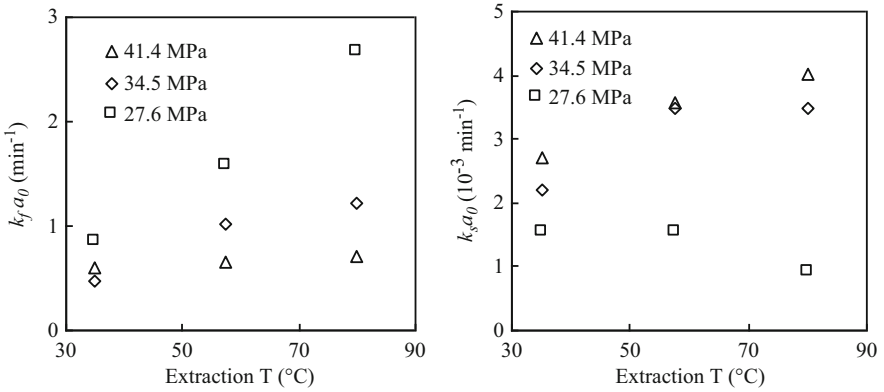

Fig. 3.3 Effects of pressure and temperature on $k_s a_0$ and $k_f a_0$ obtained using the BIC model for the $SCCO_2$ extraction of oil from fried chipped potatoes (data from Wagner et al. (2013))

increase in driving force and convection and subsequent decrease in mass transfer resistance for both fluid and solid phases. On the other hand, these two parameters are smaller for smaller particle dimension since a_0 turns to be larger and the diffusion path length becomes shorter for smaller particle size. Concerning the extraction pressure, its effect is a little bit more complicated as the $k_s a_0$ is seen to increase with pressure but the $k_f a_0$ is found to decline with pressure. Usually, the increase of pressure will promote the driving force in the $SCCO_2$ phase due to the solute solubility increment. However, the solute diffusivity in the fluid phase decreases at high pressures (Rezaei and Temelli 2000), consequently leading to an increase in mass transfer resistance (Jia et al. 2009; Döker et al. 2004). Similarly, the same pressure effect on $k_f a_0$ and $k_s a_0$ has been observed for $SCCO_2$ extraction of oil from fried chipped potatoes (Wagner et al. 2013) and $SCCO_2$ extraction of lipids from corn distiller's dried grains (Ciftci et al. 2012), as shown in Fig. 3.3.

3.2.2 Modifications of the BIC Model

Since 1994, the BIC model has proved to be successful in modeling the overall $SCCO_2$ extraction curve and has provided a powerful tool for scaling-up SFE designs (Mezzomo et al. 2009; García-Risco et al. 2011). There are two reasons for that: On the one hand, the model has provided a simplified analytical solution, which is convenient for performing the model parameters calculations. On the other hand, the broken and intact cell hypothesis has been verified by SEM analysis of botanical micro-structures (Reverchon and Marrone 2001) for many oil-rich examples where the broken cells have been identified successfully on the particle surfaces of the plant matrices. Indeed, the BIC model (Table 3.1) is suitable for the case of plug flow SFE processes without any solute-matrix interaction where the

solute accumulation and/or the solute axial dispersion D_l in the supercritical phase are assumed to be negligible.

For these reasons, a number of studies (e.g., del Valle et al. (2004), Grosso et al. (2010)) have modified the BIC model by considering the axial dispersion; then Eq. (3.2) may be rewritten as the following:

$$\rho_f u \frac{\partial y}{\partial z} + \rho_f \varepsilon \frac{\partial y}{\partial t} - \rho_f \varepsilon D_l \frac{\partial^2 y}{\partial z^2} = f(x, y) \tag{3.2a}$$

del Valle et al. (del Valle et al. 2004), who performed a numerical modeling of the $SCCO_2$ extraction of pretreated rosehip seeds, found that D_l increases with increase in CO_2 mass flow rate at laboratory scale and that at pilot plant scale the extraction becomes slow and the solute dispersion is increased between the extraction and separation vessels.

Moreover, Louli et al. (Louli et al. 2004) have taken into account the solute accumulation in the fluid phase, resulting in the following expression:

$$f(x \le x_u, y) = k_s a_0 \rho_s x \left(1 - \frac{y}{y_s}\right) \tag{3.4a}$$

Then the authors attempted modeling the $SCCO_2$ extraction of parsley seed oil, by numerically solving the modified BIC model equations with a fourth order Runge–Kutta method. However, it was found that the accumulation term seems not to significantly affect the description of SFE process.

On the other hand, Patel et al. (Patel et al. 2011) reformulated the three stage BIC model as a two-stage BIC model, thus greatly simplifying the computations with two adjustable parameters only. By incorporating the transition stage into the diffusion-controlled stage, Eqs. (3.6b) and (3.6c) can be combined together as:

$$e = x_0 - \exp\left[(q - q_{CER})\frac{k_s a_0}{\dot{q}(1-\varepsilon)}\right] \times \left[x_0 - q_{CER} \cdot y_s\left(1 - e^{-Z}\right)\right] \quad \text{for } q \ge q_{CER} \tag{3.6d}$$

The authors then applied the simplified model for simulating the $SCCO_2$ extraction of high oil containing cashew nut shells and low oil-containing black pepper. Satisfactorily, the model demonstrated high accuracy for the seeds having both high as well as low initial oil contents, and better agreement with the experimental results if compared with the original BIC model.

Recently, Sovová (Sovová 2005) generalized the original BIC model with a full consideration of solute–solid matrix interactions. This modified version is more suitable for SFE description as it takes into account solute–matrix interaction in terms of fluid–solid phase equilibrium and solvent flow pattern such as plug flow and flow with axial dispersion for longer extraction beds, and ideal mixer for short

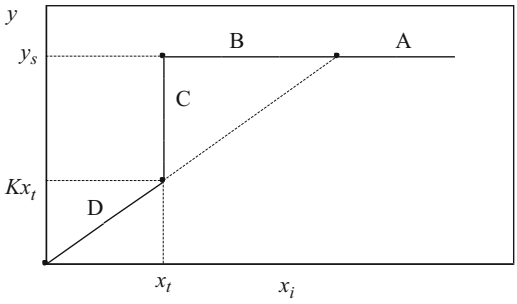

Fig. 3.4 Solute concentration curve of fluid–solid phase equilibrium, where letters A–D indicate the regions of initial equilibrium concentrations that define four types of extraction curves (Sovová 2005)

extraction beds. Thus, this modification can be advantageously applied to any kind of herbaceous material, and to SCF extraction of both essential and fatty oils.

According to this work (Sovová 2005), four types of extraction curves (A–D) are defined with respect to the initial solute composition of solid and fluid phases. Figure 3.4 depicts the discontinuous phase equilibrium between the fluid and the solid phases with broken cells. The discontinuity occurs at the assumed transition concentration, x_t, which is equal to matrix capacity for interaction with the solute. At solid–phase concentrations, lower than x_t, all of the solute interacts with the matrix and, hence, phase equilibrium is determined by partition coefficient, K. At concentrations higher than x_t, the solid phase contains also free solute whose equilibrium fluid–phase concentration is equal to the solubility, y_s.

$$y(x_b) = y_s \quad \text{for } x_b > x_t \tag{3.8a}$$

$$y(x_b) = Kx_b \quad \text{for } x_b \le x_t;\ Kx_t < y_s \tag{3.8b}$$

Before the $SCCO_2$ extraction starts, the solute equilibrium between the fluid and the solid with broken cells is supposed to be established. As the solute concentration in the untreated material is equal to that in intact cells (x_u), the initial mass balance for the solute in broken cells ($x_{b,0}$) and in the solvent (y_0) can be given as:

$$x_u - x_{b,0} = \frac{\rho_f \varepsilon}{\rho_s(1-\varepsilon)} \cdot \frac{y_0}{r} = \frac{\gamma}{r} y_0 \quad \text{for } t = 0 \tag{3.9a}$$

$$y_0 = y(x_{b,0}) \quad \text{for } x_{b,0} \ne x_t;\ x_{i,0} = x_u \tag{3.9b}$$

where $\gamma = \rho_f \varepsilon / [\rho_s(1-\varepsilon)]$ is solvent-to matrix ratio in the bed (g solvent/g solute free solid).

Rearranging Eqs. (3.8)–(3.9) according to Fig. 3.4, the initial solute concentrations can be divided into three sections:

$$\text{Sections A + B}: \quad y_0 = y_s,\ x_{b,0} = x_u - \frac{\gamma}{r} y_s \quad \text{for } x_u - x_t \ge \frac{\gamma}{r} y_s \tag{3.10a}$$

$$\text{Section C}: \quad y_0 = \frac{r}{\gamma}(x_u - x_t), \; = y_s, \; x_{b,0} = x_t \quad \text{for } \frac{\gamma}{r}Kx_t < x_\mathrm{u} - x_t < \frac{\gamma}{r}y_s \tag{3.10b}$$

$$\text{Section D}: \quad y_0 = Kx_{b,0}, \; x_{b,0} = \frac{rx_u}{r + \gamma K} \quad \text{for } x_\mathrm{u} - x_t \leq \frac{\gamma}{r}Kx_t \tag{3.10c}$$

Note that sections A and B in the equilibrium curve are divided by the point $Kx_{\mathrm{b},0} = y_\mathrm{s}$ and then $Kx_{\mathrm{b},0} \geq y_\mathrm{s}$ is satisfied for section A. Besides, for section C, where $x_{\mathrm{b},0} = x_\mathrm{t}$, the initial content of free solute is not enough to saturate the solvent in the extraction unit. Thus, four regions of initial concentrations, as indicated in Fig. 3.4 by letters A–D, can be described by Eq. (3.9). These four regions usually correspond to four different types of extraction curves.

Eqs. (3.1), (3.2), (3.8) and (3.10) can be solved numerically using Runge–Kutta method by assuming that the continuous extraction bed is represented by a series of n mixers where $n = 1$ is for ideal mixer and $n = 10$ for plug flow (Sovová 2005). For assisting the above numerical simulation, the following evaluation procedure is recommended:

1. Identifying the extraction type based on Fig. 3.4
2. Determining the model parameters y_s, x_t, K, $k_\mathrm{s}a_\mathrm{s}$ and r using the approximate model given in Eqs. (3.11)–(3.14) (Table 3.2) for different types of extraction curves
3. Optimizing the mass transfer parameters through the numerical simulation given above
4. Evaluating $k_f a_0$, eventually n from extraction curves measured at higher external mass transfer resistance

Given in Table 3.2 are the approximate models derived for evaluating the four diffident extraction curves as described in Fig. 3.4. Note that Q and N_m are the mass flow rate of the $SCCO_2$ solvent ($Q = F \times \rho_f$, g/s) and the solid charge in the extraction bed (g solute − free solid bed), respectively. a_s is specific surface area between the region of intact and broken cells ($a_s = a_0(1 - r)$, m^{-1}). C_1 and C_2 are the two BIC approximate model parameters as given in Eqs. (3.11)–(3.14).

For Type A, $x_t = 0$ is accepted for all the curves without solute–matrix interaction, subsequently resulting in an approximate solution. Furthermore, it was demonstrated that the results obtained for almond seeds oil extraction, using the original BIC model given in Table 3.1, and the model of Eq. (3.11) for Type A, are practically identical (Sovová 2005). However, it was shown that in case of solute–matrix interaction, the approximate model equations are less accurate than those of Type A case when simulating CO_2 extraction of essential oil from orange peels (Type C) or pennyroyal essential oil from leaves and flowers (Type D). Therefore, the estimated parameters should only be used as input data in the evaluation with numerical simulations rather than as final results.

Table 3.2 The BIC approximate models used for identifying extraction curves

Extraction curves	Solute–matrix interaction	Approximate model	No.
Type A	$x_t = 0$ $r = 1 - C_1 \cdot \exp(-C_2 q_{CER}/2)$ $k_s a_s = (1-r)(1-\varepsilon)QC_2/N_m$	$e = qy_s \quad \text{for } 0 \le q \le q_{CER}$ $e = x_u[1 - C_1 \exp(-C_2 q)] \quad \text{for } q > q_{CER}$	(3.11)
Type B	$x_t > 0$ $r = 1 - C_1 \cdot \exp(-C_2 q_{CER})$ $k_s a_s = \dfrac{(1-r)(1-\varepsilon)QC_2}{N_m[1-(1-r)C_2/K]}$ $k_s a_s = k_s a_0 (1-r)$	$e = qy_s \quad \text{for } 0 \le q \le q_b = \dfrac{r(x_u - x_t) - \gamma \cdot K \cdot x_t}{y_s - K \cdot x_t}$ $e = q_1 \cdot y_s + (q - q_b)Kx_t \quad \text{for } q_b \le q \le q_{CER}$	(3.12)
Type C		$e = q\dfrac{r(x_u - x_t)}{\gamma} = qy_0 \quad \text{for } 0 \le q \le \gamma$ $e = r(x_u - x_t) + (q - \gamma)Kx_t \quad \text{for } \gamma \le q \le q_{CER}$	(3.13)
Type D		$e = q\frac{Kx_u}{1+K(\gamma/r)} = qy_0 \quad \text{for } 0 \le q \le q_{CER}$	(3.14)

The new modification seems to be practically meaningful as it is able to simulate different types of extraction curves and characterize them with mutually comparable parameters as mass transfer coefficients and equilibrium constants. Interestingly, it is particularly suited to fit experimental data as it almost independently simulates two extraction periods, the first one governed by phase equilibrium and the second one governed by the internal diffusion in particles. Thus, not only a numerical solution to the more generalized model was given but also a simplified approximate model for describing the overall extraction yield curves was proposed (Sovová 2005). Moreover, the approximate model for extraction curves of Type A is compatible with the original BIC model and both should be applied only when there is no solute–matrix interaction.

The new version is more general than the original one but its application to extraction processes simulation is quire complicated as it involves a number of model parameters such as 1–3 for phase equilibrium, 2–3 for mass transfer, and 0–1 for flow pattern. As such, the applications of the new BIC version are less frequently reported in open communications (Louli et al. 2004; Sovová et al. 2010; Martín et al. 2011; Rebolleda et al. 2012; Mouahid et al. 2013). Furthermore, Sovová et al. (Sovová et al. 2010) have attempted the new version of the BIC model with solute–solid interactions for describing minor-component extraction of vegetable substrates but found that the model turns to be invalid for the cases of extraction of essential oils that are located only inside the matrix. Obviously, essential oils from intact matrices become less accessible and the extraction process turns to be diffusion-controlled and much slow due to high mass transfer resistance. In this sense, the first part of equilibrium-controlled extraction can not apply if following the broken-and-intact-cells hypothesis.

More recently, Sovová and Stateva (Sovová and Stateva 2011) have shown that the appropriate model for $SCCO_2$ extraction from vegetable materials can be properly chosen if based on characteristic times of individual extraction steps. Sovová (Sovová 2012) has further integrated simplified equations of extraction curves with characteristic times of four single extraction steps: internal diffusion, external mass transfer, hypothetic equilibrium extraction without mass transfer resistance, and displacement of the solution from the extractor. The evaluation of experimental extraction curves using these equations can facilitate the choice of proper detailed model for SFE and enable estimation of changes in the extraction kinetics with the changes in operation conditions and extraction geometry.

3.3 The Shrinking Core (SC) Model

3.3.1 Original Model

The shrinking core (SC) model, adopted from Goto et al. (Goto et al. 1996), has been used to describe irreversible desorption followed by diffusion in the porous solid particles through the pores. According to this model, as the extraction

proceeds, a sharp boundary of the particle core where the pores are still filled with the solute moves to particle center, while the pores between the core and particle surface are filled with supercritical fluid. The solute–fluid interface is in the pores on the core surface, and the equilibrium concentration in the fluid at the interface is equal to the solubility of the solute in the supercritical fluid. The mass transfer resistance in the pores between the core and particle surface, which increases as the core shrinks, is assumed to control the extraction rate. However, the shrinking core model is only rarely applied to model SFE of essential oils because it assumes that the pores of spherical particles are initially completely filled with the solute but the content of essential oils in plants is usually not sufficient to fill the pores.

The SC model has involved a number of assumptions such as isothermal and isobaric system, constant physical properties of the $SCCO_2$ fluid during extraction, irreversible desorption process and negligible radial dispersion. Axial dispersion can be neglected to simplify the analysis although it is considered in most cases. Based on the assumptions and considering axial dispersion, the dimensionless material balances in the fluid and solid phases are described as Eqs. (3.15) to (3.16), respectively (refer to Table 3.3). The diffusion in the outer shell and the average solute concentration within the core can be readily given as Eqs. (3.17) to (3.18), respectively. Coupled with initial and boundary conditions, and two Danckwerts boundary conditions at the extraction unit inlet and outlet, the differential mass balance equations can be finally solved without considering the solute–solid interaction, but resulting in a rather complicated implicit solution (see Eq. (3.20)) for the overall extraction curve.

In Table 3.3, C is the dimensionless concentration in the bulk fluid phase ($C = c/c_{sat}$); C_i is the dimensionless concentration in the pores ($C_i = c_i/c_{sat}$); c is the solute concentration in the bulk $SCCO_2$ phase (g/m^3); c_i is the solute concentration in the pores within particles (g/m^3); $c_i(R)$ is the solute concentration in pores at the particle surface (g/m^3) and c_{sat} is the concentration of the solute that is in equilibrium at a solid surface ($c_{sat} = y_s\rho_f$, g/m^3). Λ is the dimensionless solid phase concentration ($\Lambda = c_s/c_{s0}$), where c_s and c_{s0} are respectively the solute concentration and initial solute concentration in the solid phase in unit of g/m^3, and $\overline{\Lambda}$ is dimensionless averaged solute concentration in the solid phase. Z is the dimensionless axial coordinate ($Z = z/H$); z is the axial coordinate along the bed in the extraction unit (m) and H is the height of the packed extraction bed. ξ is the dimensionless radial coordinate in the particle ($\xi = r/R$) and ξ_c is the dimensionless radial coordinate at the shrinking core surface ($\xi_c = r_c/R$); r is the radial coordinate within the particle (m); r_c and R are the radius of a shrinking core and that of the spherical solid particle in unit of m, respectively. θ is the dimensionless extraction time ($\theta = D_e t/R^2$); t is the extraction time (s) and D_e is the effective diffusion coefficient (m^2/s). ω is a dimensionless constant parameter ($\omega = c_{sat}/c_{s0}$) and σ is a dimensionless model parameter ($\sigma = uR^2/D_eH$). B_i is the dimensionless Biot number ($B_i = 2Rk_f/D_e$), where k_f is film mass transfer coefficient (m/s). P_e is the dimensionless Peclet number ($P_e = Hu/D_l$), where u is the superficial velocity of the

Table 3.3 Details for the dimensionless SC model

Equation	No.
Dimensionless differential mass transfer in the solid phase:	
$\frac{\partial \overline{\Lambda}}{\partial \theta} = 3B_i\omega[C - C_i(\xi = 1)]$	(3.15)
Dimensionless differential mass transfer in the bulk fluid phase	
$\frac{\partial C}{\partial \theta} + \sigma \frac{\partial C}{\partial Z} = \frac{\sigma}{P_e}\frac{\partial^2 C}{\partial Z^2} - 3B_i\frac{1-\varepsilon}{\varepsilon}[C - C_i(\xi = 1)]$	(3.16)
Dimensionless diffusion in the outer shell region	
$\frac{1}{\xi^2}\frac{\partial}{\partial \xi}\left(\xi^2 \frac{\partial C_i}{\partial \xi}\right) = 0 \quad \text{or} \quad C - C_i(\xi = 1) = \frac{C-1}{1-B_i(1-1/\xi_c)}$	(3.17)
Dimensionless solid phase solute concentration within the core	
$\overline{\Lambda} = \xi_c^3$	(3.18)
Dimensionless initial conditions	
at $\theta = 0$, $\xi_c = 1$, $\Lambda = 1$ and $C = 0$	(3.19a)
Dimensionless boundary conditions	
$C_i = 1 \quad \text{at} \quad \xi = \xi_c$ $\left(\frac{\partial C_i}{\partial \xi}\right)_{\xi=1} = B_i[C - C_i(\xi = 1)]$	(3.19b)
Dimensionless Danckwerts boundary conditions at the extraction unit inlet and outlet	
$C - \frac{1}{P_e}\frac{\partial C}{\partial Z} = 0 \quad \text{at} \quad Z = 0, \quad \frac{\partial C}{\partial Z} = 0 \quad \text{at} \quad Z = 1$	(3.19c)
Solution	
$E = 1 - \Lambda = \frac{\sigma\omega\varepsilon}{1-\varepsilon}\int_0^\theta C d\theta$	(3.20a)
$C = 1 - \exp\left[-\frac{1-\varepsilon}{\sigma\varepsilon} \cdot \frac{3B_iZ}{1-B_i(1-1/\xi_c)}\right]$	(3.20b)
$\theta = \frac{Z}{\sigma} + 1 - \frac{1}{\omega B_i}\int_1^{\xi_c} \frac{\xi_c^2[1 - B_i(1 - 1/\xi_c)]}{\exp\{[(\varepsilon - 1)/\sigma\varepsilon] \cdot 3BiZ/[1 - B_i(1 - 1/\xi_c)]\}} d\xi_c$	(3.20c)
$\xi_c^3 = 1 - \frac{3\omega B_i(\theta - Z/\sigma)}{1-B_i(1-1/\xi_c)}\exp\left[-\frac{1-\varepsilon}{\sigma\varepsilon} \cdot \frac{3B_iZ}{1-B_i(1-1/\xi_c)}\right]$	(3.20d)

$SCCO_2$ solvent (m/s) and D_l is the solute axial dispersion coefficient in the $SCCO_2$ solvent (m^2/s).

The parameters involved in the model are the mass transfer coefficient in the bulk phase k_f, the axial dispersion coefficient in the fluid phase D_l, the effective pore diffusivity in the particles D_e, and the solute solubility y_s in $SCCO_2$ (that has been extensively discussed in previous section). Usually, the k_f parameter can be estimated by using a number of empirical correlations involving a few dimensionless numbers (del Valle and De La Fuente 2006; Oliveira et al. 2011; Huang et al. 2012). For example, $S_h = 2.0 + 1.1R_e^{0.6}S_c^{1/3}$ and $S_h = d_p k_f/D_{12}$, $R_e = \rho_f u d_p/\mu$, $S_c = \mu/\rho_f D_{12}$. S_h, R_e, and S_c are the dimensionless numbers of Reynolds, Sherwood, and Schmidt, respectively. μ is the viscosity of the supercritical solvent phase (g/m · s) and is readily evaluated from available experimental data. D_{12} is the external diffusion coefficient (m^2/s) of the solute to the supercritical fluid and its values may be similarly obtained from existing correlations (Oliveira et al. 2011). As for D_l, it can be simply assumed to be equal to zero or regressed against experimental data.

Usually it is conveniently estimated with certain correlations such as $\varepsilon D_l/D_{12} = 1.317(\varepsilon R_e S_c)^{1.392}$ for $\varepsilon R_e S_c > 0.3$ (Oliveira et al. 2011). Sometimes, a dimensionless Peclet number is suggested to evaluate the importance of axial dispersion, i.e. $Pe = Hu/D_l$.

Much earlier, Tan and Liou (Tan and Liou 1989a) have systematically investigated the axial dispersion of supercritical carbon dioxide in packed beds by injection of a pulse of methane into $SCCO_2$. Their data show that the axial dispersion coefficient D_l increases with the interstitial velocity u and packed particle diameter d_p, and that it also increases with the density and viscosity of $SCCO_2$. Similarly, D_l is found to decrease as extraction pressure decreases or temperature increases. The bed height offers a negligible effect on the axial dispersion if $H(1-\varepsilon)/(\varepsilon d_p P_e) \geq 0.3$ is satisfied and the Peclet number obtained is in the range of 0.6–2.7. The authors have also observed that the correlations in terms of Peclet, Reynolds and Schmidt dimensionless groups do not offer satisfactory prediction. However, a number of studies (del Valle and De La Fuente 2006; Oliveira et al. 2011; Huang et al. 2012; Goto et al. 1996; Reverchon and Marrone 2001) have addressed that, practically, the axial dispersion may have little effect on $SCCO_2$ extraction as P_e or P_e/ε is generally larger than 100, due to the fact that the typical size of solid particles loaded in the bed is very small as compared to the diameter of the extraction unit (ratio less than 0.1). In this case the axial dispersion problem may have been avoided during SFE process.

As for D_e, it is usually taken as an adjustable parameter and directly regressed from the experimental extraction data. It is unsurprising that the simulation work with the SC model solution, i.e., Eq. (3.20), must be solved numerically (Salgın et al. 2006; Ajchariyapagorn et al. 2009; Machmudah et al. 2006; Balachandran et al. 2006; del Valle et al. 2006; Tezel et al. 2000; Ahmed et al. 2012; Bashipour and Ghoreishi 2012). Salgın et al. systematically investigated the influences of extraction pressure, temperature, particle size and CO_2 flow rate on the extraction of sunflower oil with $SCCO_2$ (Salgın et al. 2006). Their results show that both k_f and D_e are increasing with extraction pressure, temperature and CO_2 flow rate but either the increase in CO_2 flow rate or the decrease in the extraction pressure can lead to a reduction of D_l. However, Ajchariyapagorn et al. (Ajchariyapagorn et al. 2009) who applied the SC model for simulating $SCCO_2$ extraction of nimbin from neem seeds, demonstrate that an increase in pressure leads to a decrease in both k_f and D_e values, and that D_l increases as CO_2 flow rate increases.

Tezel et al. (Tezel et al. 2000) further extended the SC model to $SCCO_2$ extraction of two pseudo-component systems from porous matrix. Their simulations were carried out for three different cases where either the two components were homogeneously distributed within the host matrix, or only the heavier component was in the outer core, or just the lighter component constituted the outer core as an initial condition. With tomato seed extraction as a reference, the multicomponent SC model can predict selective extraction of the lighter and heavier fatty acids by $SCCO_2$ extraction.

Later, Machmudah et al. (Machmudah et al. 2006) successfully used the SC model for simulating $SCCO_2$ extraction of nutmeg seeds. Their computer

simulations gave different D_e values for the lighter and heavier components, ranging from $(4.33 \times 10^{-9}$ to $7.69 \times 10^{-8})$ m^2/s and from $(1.90 \times 10^{-9}$ to $3.20 \times 10^{-8})$ m^2/s, respectively. Furthermore, the calculations showed that the B_i values, from 14.9 to 107, were significantly larger than 10, indicative of the importance of the internal mass transfer resistance in comparison to the external mass transfer resistance. However P_e, spanning from 7.5 to 26.3, is far less than 100, contrary to most cases of $SCCO_2$ extraction. This suggests that the axial dispersion impact on the mass transfer rate is rather pronounced and cannot be neglected in this case.

In addition, del Valle et al. (del Valle et al. 2006) have introduced a new term, microstructural correction factor, as the ratio between the porosity and tortuosity of the particle itself to quantitatively relate the effective diffusivity (D_e) inside the porous particles with the oil diffusivity (D_{12}) in $SCCO_2$. In order to study the effects of pretreated seed microstructures on extraction kinetics and mass transfer coefficients del Valle et al. (del Valle et al. 2006) applied the microstructure—extractability relationships for modeling $SCCO_2$ extractions of prepressed rapeseeds, olive husks, and flaked rosehip seeds using the SC model.

Similarly, Balachandran et al. (Balachandran et al. 2006) investigated the effects of sample drying and sample water content on the supercritical extraction of oleoresin from ginger and found that water-rich fresh ginger resulted in high extraction yields and high mass transfer rate as well.

3.3.2 Modifications of the SC Model

From the viewpoint of engineering application, one prefers a simpler model rather than an exact but complicated model. In fact, the SC model can be simplified for easily simulating the $SCCO_2$ extraction process involving strong solute–solid interaction and negligible axial dispersion. In this case, the SC model should consider the solute equilibrium in the solid phase and within the pores. Thus, the total initial solute concentration in porous solid particles, c_{s0} (g/m^3), is either composed of solute adsorbed on solid sites, c_{a0} (g/m^3), or is free within the pores, c_{i0} (g/m^3), as shown in Eq. (3.21). One may note that the porosity of the solid particle, ε_p, is assumed to be constant in the extraction process. If fast adsorption/desorption process can be assumed, the interaction of the solute with the fluid and solid phases may be linearly expressed as Eq. (3.22), with the term of K as the equilibrium coefficient between the solute in the fluid and solid phases (Goto et al. 1993).

By considering a parabolic concentration profile in the solid particles, the internal and external mass transfer processes can be combined and described by a linear driving force approximation (Goto et al. 1993), i.e., Eq. (3.23). In the meantime, the overall mass transfer coefficient for a spherical particle, k_p may be given by Eq. (3.24).

Table 3.4 The simplified SC model with analytical solution

Equation	No.
Initial solute concentration in a particle: $c_{s0} = (1-\varepsilon_p)c_{a0} + \varepsilon_p c_{i0}$	(3.21)
Linear interaction between solute and solid: $c_s = Kc_i$	(3.22)
Linear driving force approximation	
$\frac{k_f}{R}\frac{1-\varepsilon}{\varepsilon}[c - c_i(R)] = \frac{15D_e}{R^2}(1-\varepsilon)[c_i(R) - c_i]$	(3.23)
Overall mass transfer coefficient for a spherical particle	
$k_p = \frac{k_f}{1+B_i/5}$	(3.24)
Differential mass transfer in the fluid phase:	
$\frac{\partial c}{\partial t} + u\frac{\partial c}{\partial z} = -\frac{1-\varepsilon}{\varepsilon}\frac{3k_f}{R}[c - c_i(R)]$	(3.25a)
$\varepsilon\frac{\partial c}{\partial t} + \frac{c}{\tau} = \frac{3k_p}{R}(1-\varepsilon)\left(\frac{c_s}{K} - c\right)$	(3.25b)
Differential mass transfer in the solid phase:	
$\varepsilon_p\frac{\partial c_i}{\partial t} + (1-\varepsilon_p)\frac{\partial c_s}{\partial t} = \frac{D_e}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial c_i}{\partial r}\right)$	(3.26a)
$\left[\frac{\varepsilon_p}{K} + (1-\varepsilon_p)\right]\frac{\partial c_i}{\partial t} = \frac{3k_p}{R}\left(c - \frac{c_s}{K}\right)$	(3.26b)
Analytical solution	
$c = \frac{\psi(1-\varepsilon)c_{s0}}{\varepsilon[\varepsilon_p(1-\varepsilon_p)K](m_1-m_2)}(e^{m_1 t} - e^{m_2 t})$	(3.27a)
$E = \frac{\psi(1-\varepsilon)}{\varepsilon[\varepsilon_p+(1-\varepsilon_p)K](m_1-m_2)}\left[\frac{e^{m_1 t}-1}{m_1} - \frac{e^{m_2 t}-1}{m_2}\right]$	(3.27b)
$\psi = k_p a_p \tau, \quad \tau = D_e H/uR^2, \quad \Phi = \frac{\psi}{\varepsilon[\varepsilon_p+(1-\varepsilon_p)K]}$	(3.28a)
$\Gamma = \frac{1}{\varepsilon} + \frac{\psi(1-\varepsilon)}{\varepsilon} + \frac{\psi}{\varepsilon_p+(1-\varepsilon_p)K}$	(3.28b)
$m_1 = \frac{-\Gamma+\sqrt{\Gamma^2-4\Phi}}{2}, \quad m_2 = \frac{-\Gamma-\sqrt{\Gamma^2-4\Phi}}{2}$	(3.28c)

Differential mass transfer equations in the fluid phase and solid phase can be initially given as Eqs. (3.25a) and (3.26a) and further reduced to Eqs. (3.25b) and (3.26b), respectively, with the aid of Eqs. (3.22)–(3.24). Eqs. (3.25b) and (3.26b) can be exactly solved with a simple explicit analytical solution, as given by Eq. (3.27a) and (3.27b).

The details of this simplified SC model are given in Table 3.4. Obviously, the simpler the SC model is the easier is to use it and, thus, it has been applied by a lot of researchers to simulate the $SCCO_2$ extraction of various botanical materials (Tonthubthimthong et al. 2004; Mongkholkhajornsilp et al. 2005; Goto et al. 1993; Kitzberger et al. 2009).

Tonthubthimthong et al. (Tonthubthimthong et al. 2004) successfully applied this simple SC model for $SCCO_2$ extraction of nimbin from neem seeds. Their calculations show that the extraction yield depends strongly on the solvent flow rate, and that the external mass transfer or equilibrium is the process controlling step. In conjunction with these extraction results, Mongkholkhajornsilp et al. (Mongkholkhajornsilp et al. 2005) proposed a new correlation among dimensionless numbers of Sherwood, Reynolds and Schmidt with the view to improve the model performance. Superior simulation results were obtained with the correlation

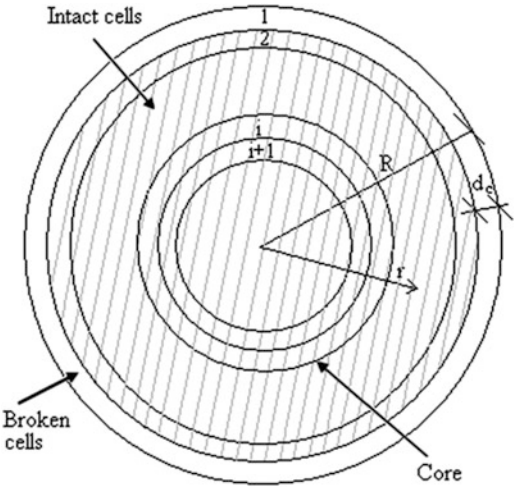

Fig. 3.5 Particle scheme for the BIC plus SC model

$S_h = 0.135 R_e^{0.5} S_c^{0.33}$ optimized against experimental data of nimbin extraction. Interestingly, Machmudah et al. (Machmudah et al. 2006), who recently compared the performances of the BIC and SC models for $SCCO_2$ extraction of nutmeg oil, found that the SC model could describe the experimental data for all extraction conditions well whereas the BIC model with discontinuous phase equilibrium between fluid and solid phases (Sovová 2005) could only describe the data at lower extraction yields.

For these reasons, Fiori et al. (Fiori et al. 2009) attempted to bridge the BIC and SC models by proposing a very interesting novel relation which combines the concepts of both models. Figure 3.5 graphically shows the BIC plus SC model where the milled solid particle can be divided into a series of concentric spherical shell layers consecutively numbered starting from the external one.

On the one hand, the proposed model takes into account the seed inner structure with the presence of oil-bearing cells. Then the ground seed particles can be assumed as spherical particles formed by a number of concentric shells. Each shell may only consist of an oil–bearing cell layer and the total number of concentric layers in a particle, N_c, is defined as the ratio of particle radius R to cell diameter d_c (d_c could be measured by SEM). On the other hand, during the extraction process, the oil content is irreversibly and progressively exhausted, in sequence from the outside layer consisting of broken cells to the inside layer consisting of intact cells, with a core shrinking during the process. Thus, the retraction of the core increases the mass transfer resistance to the fluid phase because the solute has to diffuse across each depleted layer. For this reason, Fiori et al. (Fiori et al. 2009) expressed doubts whether the concentration inside the core might be higher than the solute solubility in the $SCCO_2$ fluid. Details of BIC plus SC model are given in Table 3.5.

As seen from Fig. 3.5, the external mass transport refers to the outer layer of the particles from which the so-called free oil is released and this process is governed

Table 3.5 Details for the BIC plus SC model

Equation		No.
Overall oil mass balance for the whole particle		
$\frac{\partial\overline{\Omega}}{\partial t} = \frac{3k_p}{R}\frac{c_{sat}-c}{c_{s,0}}$		(3.29)
Differential mass transfer in the fluid phase:		
$\frac{\partial y}{\partial t} + \frac{u}{1-\varepsilon}\frac{\partial y}{\partial z} - D_l\frac{\partial^2 y}{\partial z^2} = \frac{1}{\varepsilon}k_p a_0(y_s - y)$		(3.30)
Initial and boundary conditions:	$\begin{cases}\Omega(z,t=0)=\Omega_0=\dfrac{\varepsilon\cdot c_{sat}}{(1-\varepsilon)c_{s,0}}\\ y(z=0,t)=0\\ y(z>0,t)=y_s\\ \left.\frac{\partial y}{\partial z}\right\|_{z=H,t}=0\end{cases}$	(3.31)
Overall mass transfer coefficient, and average solute concentration for spherical particles		
Discrete	$\frac{1}{k_{p,j}} = \frac{1}{k_f} + \frac{1}{k_c}\sum_{j=1}^{Nc-1}\left(\frac{R}{R-jd_c}\right)^2, \quad 1 \le j \le N_c - 1$	(3.32a)
	$\Omega_j = 1 - \left(\frac{R-jd_c}{R}\right)^3, \quad 1 \le j \le N_c - 1$	(3.32b)
Semi-continuous	$\frac{1}{k_p(r)} = \frac{1}{k_f}, \quad R - d_c \le r \le R$ $\frac{1}{k_p(r)} = \frac{1}{k_f} + \frac{1}{k_c}\frac{R-r}{d_c}\frac{R}{r}, \quad 0 \le r \le R - d_c$	(3.33a)
	$\Omega_r = 1 - \left(\frac{r}{R}\right)^3, \quad 0 \le r \le R$	(3.33b)
Continuous	$\frac{1}{k_p(r)} = \frac{1}{k_f} + \frac{1}{k_c}\cdot\frac{R-r}{d_c}\cdot\frac{R}{r}, \quad 0 \le r \le R$	(3.34a)
	$\Omega_r = 1 - \left(\frac{r}{R}\right)^3, \quad 0 \le r \le R$	(3.34b)

by the external mass-transport coefficient, k_f. Thus, the outer layer extraction will be restrained by the grinding conditions. As known from experimental observations, the mass transport coefficient for entire seeds or badly ground seeds is usually very small, while for finely ground seeds the mass transport coefficient will span in a broader range, depending on the degree of grinding.

When the solute in the outer shell is emptied, extraction from the second shell starts in two subsequent stages: one related to the septum that separates the first shell from the second shell, and the other equivalent to the external mass transport. As the extraction proceeds, the particle can be schematized as being composed of external solute–emptied shells filled with $SCCO_2$, one shell from which the oil is being extracted, and the oil-filled inner shells. Note that the mass transfer resistance inside the cell may be negligible when compared to that of the outer-cell septa and thus the overall mass transport coefficient does not vary notably during the shell emptying.

Clearly, as seen in Table 3.5, the internal mass transport coefficient can be described with three alternative models: (a) Employing a continuous function in all particles to calculate the dependence of the coefficient with core radius—continuous model; (b) Using a constant coincident with k_f until the first layer is

exhausted and then applying a continuous function of the core radius—semi-continuous model; (c) Using a constant value of the coefficient for each layer—discrete model.

In the BIC plus SC model, the authors (Fiori et al. 2009) have defined a new parameter of the solute exhaustion degree of the particle, Ω, as the ratio between the exhausted volume and the total volume. Then the time-dependence of this parameter can be used to evaluate the oil mass balance for the whole particle, i.e., Eq. (3.29) in Table 3.5. In the equations given in Table 3.5, k_c is defined as the inner-shell mass transfer coefficient (m/s), j is the layer number; Ω_j and $k_{p,j}$ are the exhaustion degree of the j^{th} spherical shell and the overall mass-transfer coefficient of the j^{th} shell, respectively. Similarly, Ω_r and $k_p(r)$ are the radial coordinate position dependent exhaustion degree and the overall mass-transfer coefficient in the particles.

As seen in Table 3.5, the first term on the right hand side of Eqs. (3.32a), (3.33a) and (3.34a) is the mass transfer resistance in the first shell, i.e., convective mass transfer coefficient, while the second term represents the resistance added as each shell is depleted. According to Fiori et al. (Fiori et al. 2009), the resistance in the first layer at the particle surface is lower due to the presence of broken cells containing free oil as observed by SEM. After the broken cells in the first layer have been depleted of free oil, the extraction of the intact cells in the inner layers is initiated. The layers composed of intact cells must have higher resistance to mass transfer due to the presence of cell walls, as reflected by decreasing the pseudo mass transfer coefficient proportionally to the distance to the particle surface.

A rigorous comparison of the three approaches proposed has revealed that the discrete and the semi-continuous models are superior to the continuous one which undervalues the same coefficient. The BIC plus SC model has been verified by simulating the extraction of grape seed oil at 55 MPa and 313 K (Fiori 2007) and the extraction of almond oil at 35 MPa and 313 K (Marrone et al. 1998). Disagreement between the experimental and modeling results has been found, probably due to the existence of broken cells in the internal layers of the particle. In order to overcome this limitation, the first layer could be assumed to have twice the thickness of the other layers. With this assumption, the accuracy of the fitting of experimental results has been greatly improved. The results of the above studies have also shown that the model performance is heavily dependent on the real granulometric size distribution rather than on the weight-averaged particle dimension.

3.4 The Micro-Structured Mathematical Model

More recently, Zizovic and coworkers extensively attempted to apply plant physiology knowledge to the investigation of $SCCO_2$ extraction of essential oils from different plant material (Ivanovic et al. 2011; Zizovic et al. 2005, 2007a, b, 2012; Stamenić et al. 2008, 2010; Meyer et al. 2012a, b; Stamenić and Zizovic 2013; Ivanović et al. 2013). Incorporation of plant physiology knowledge about the sites

of essential oil synthesis and storage in plant material into the description of SFE of essential oils can make possible the development of a new type of theoretical mathematical models on the micro-scale scale. A thorough analysis of SEM images before and after extraction has evidenced the presence of specific secretory structures like peltate glands, secretory ducts and secretory cells/cavities in which the essential oil is stored.

Then, three stages of extraction may be distinguished: from the secretory structures disrupted during pretreatment, from the secretory structures broken after a certain time of exposure to supercritical CO_2, and the slowest extraction from intact secretory structures. Based on these observations, the authors proposed the micro-scale mathematical model (MSMM) for describing the process of $SCCO_2$ extraction of essential oils from various botanical matters.

The general equation of the MSMM model, i.e. the mass balance for the supercritical phase in the extraction unit can be written as:

$$\frac{\partial Y}{\partial t} = D_l \frac{\partial^2 Y}{\partial z^2} - u \frac{\partial Y}{\partial z} + ST \tag{3.35}$$

where ST is the Source and Transfer term which describes essential oil transfer from specific secretory structure to supercritical fluid phase. The Source and Transfer term is presented in Table 3.6 for each specific type of secretory structure (Zizovic et al. 2005, 2007a, b; Stamenić et al. 2008).

In Table 3.6, V is void volume of the extraction chamber (m^3). N_d, N_p and N_s are total number of secretory ducts, total number of peltate glands and total number of secretory cavities or cells, respectively. R_d and R_{nd} are radius of the secretory duct (m) or radius of the nondisrupted peltate gland in which the essential oil is saturated with CO_2 (m), respectively. $\Re_p$, $\Re_s$ and $\Re_w$ are assumed to be the radius of the oil sphere from disrupted peltate glands (m), radius of the oil sphere from the disrupted secretory cells (m) and radius of wetted particles (m), respectively. Here, d_c is defined as the diameter of secretory cell or cavity (m) and δ is the difference between particle radius and secretory structure radius (m). γ_s, ϕ and φ are fraction of cavities or cells disrupted by grinding, fraction of peltate glands disrupted during grinding pretreatment, and fraction of peltate glands nondisrupted during grinding but disrupted by CO_2 dissolving, respectively. t_d is the time needed when peltate glands disruption caused by CO_2 dissolving occurs (s) while t_w is the time needed when the oil that embedded the particles is extracted (s). Y, Y^e and Y_s are essential oil concentration in $SCCO_2$ phase ($kmol/m^3$), essential oil concentration in $SCCO_2$ phase at the duct end ($kmol/m^3$), and concentration of the essential oil in $SCCO_2$ at $SCCO_2$ – oil interface ($kmol/m^3$), respectively. X is the essential oil concentration in undisrupted secretory structure ($kmol/m^3$). a, a_c and a_w are respective specific surface area (m^2/m^3) of disrupted peltate gland, secretory cells or wetted particles referred to $SCCO_2$ volume. a_d and a_{nd} are respective specific surface area (m^2/m^3) of open duct ends available for mass transfer and nondisrupted peltate gland containing essential oil saturated with CO_2, referred to $SCCO_2$ volume while a_R

Table 3.6 Source and Transfer term for each type of secretory structure

Secretory structure	Source and transfer term	No.
Glandular trichomes (peltate glands)	$ST = N_p a k_f \phi (Y_s - Y)$ for $t \le t_d$	(3.37a)
	$ST = N_p a k_f \phi (Y_s - Y) + N_p (1-\phi)\varphi a k_f (Y_s - Y) + N_p (1-\varphi)(1-\phi) a_{nd} \frac{3}{R_{nd}} D_e (X - Y)$ for $t > t_d$	
	where $a_{nd} = \frac{4\pi R_{nd}^2}{V\varepsilon}$, $a = \frac{4\pi \Re_p^2}{V\varepsilon}$	
	$ST = N_p a k_f \phi (Y_s - Y) + N_p (1-\phi)\varphi a k_f (Y_s - Y) + N_p (1-\varphi)(1-\phi) a_{nd} \frac{3}{R_{nd}} D_e (X - Y)$ for each value for t and $\varphi \neq$ constant	(3.37b)
Secretory ducts	$ST = a_w k_f (Y_s - Y)$ for $t \le t_w$ $ST = a_d k_f (Y^e - Y)$ for $t > t_w$ where $a_d = \frac{2N_d \pi R_d^2}{V\varepsilon}$, $a_w = \frac{4(1\text{-}\varepsilon)}{\Re_w \varepsilon}$	(3.38)
Secretory cavities and cells for $d_c \ge d_p$	$ST = a_w k_f (Y_s - Y)$ for $t \le t_w$ $ST = N_s a_c \frac{D_e}{\delta}(X - Y)$ for $t > t_w$ where $a_c = \frac{N_s \pi d_c^2}{2V\varepsilon}$, $a_w = \frac{4(1\text{-}\varepsilon)}{\Re_w \varepsilon}$	(3.39a)
Secretory cavities and cells for $d_c < d_p$	$ST = a_R N_s \gamma_s k_f (Y_s - Y) + N_s (1-\gamma_s) a_c k_s (X - Y)$ where $a_R = \frac{4\pi \Re_s^2}{V\varepsilon}$, $a_c = \frac{N_s \pi d_c^2}{2V\varepsilon}$	(3.39b)

is defined as specific surface area (m^2/m^3) of the oil spheres from disrupted secretory cells referred to $SCCO_2$ volume.

The corresponding initial and boundary conditions are:

$$\begin{aligned} &t = 0, \quad Y = 0 \quad at \;\; 0 \le z \le H \\ &t > 0, \quad Y = 0 \quad at \;\; z = 0 \\ &t > 0, \quad \frac{\partial Y}{\partial z} = 0 \quad at \;\; z = H \end{aligned} \tag{3.36}$$

For each type of secretory structure, the differential equations, describing the secretory structures behavior during the process of SFE, need to be solved along with Eq. (3.35) and these equations are listed in Table 3.7. Note that Y^d is defined as essential oil concentration in the $SCCO_2$ phase inside the duct ($kmol/m^3$) while Y_{sat} is given as essential oil concentration in the oil sphere of disrupted secretory structure saturated with CO_2 ($kmol/m^3$).

The MSMM model can be solved numerically for each specific type of secretory structure using the explicit form of the finite difference method. It should be noted that certain parameters, such as secretory structure dimensions, are identified and introduced in order to obtain better description of the phenomenology of the SFE process (Zizovic et al. 2005). The total number of peltate glands (N) is calculated on the basis of the essential oil quantity in plant tissue and the average volume of

Table 3.7 The differential equations for the micro-structured mathematical model

Secretory structure	Differential equations	No.
Glandular trichomes (peltate glands)	$-\frac{d\mathfrak{R}_p}{dt} = \frac{k_f}{Y_{sat}}(Y_s - Y) \quad \text{for } t \le t_d$ $-\frac{dX}{dt} = \frac{D_e}{4\pi R_{nd}^2}(X - Y) \quad \text{for } t > t_d$	(3.40a)
Secretory ducts	$-\frac{d\mathfrak{R}_w}{dt} = \frac{k_f}{Y_{sat}}(Y_s - Y) \quad \text{for } t \le t_w$ $-D_{12}\frac{dY^d}{dz} = k_f(Y^e - Y) \quad \text{for } t > t_w$	(3.40b)
Secretory cavities and cells for $d_c \ge d_p$	$-\frac{d\mathfrak{R}_w}{dt} = \frac{k_f}{Y_{sat}}(Y_s - Y) \quad \text{for } t \le t_w$ $-\frac{dX}{dt} = \frac{3k_s\delta}{(d_c/2)^2}(X - Y) \quad \text{for } t > t_w$	(3.40c)
Secretory cavities and cells for $d_c < d_p$	$-\frac{d\mathfrak{R}_s}{dt} = \frac{k_f}{Y_{sat}}(Y_s - Y)$ $-\frac{dX}{dt} = \frac{3k_s\delta}{(d_c/2)^2}(X - Y)$	(3.40d)

the peltate gland. The radius of the peltate gland in which the essential oil is saturated with CO_2 (R_d) may be estimated using the cubic equation of state model. The time needed for this saturation (t_d) equals the time when peltate gland disruption, caused by $SCCO_2$ dissolving, occurs, and can be estimated from the experimental results at the point of sudden increase in the rate of the SFE due to the essential oil release caused by cracking. In the calculation, the diffusivity of $SCCO_2$ in solid matrix of unstretched peltate gland membrane has been accepted to be equal to 10^{-13} m^2/s for the entire Lamiaceae family. This value is similar to that reported in the study of Reverchon et al. (Reverchon et al. 1993) where the diffusivity related to essential oils in the solid matrix of these plants, estimated with the heat analogue model presented later, varies between (1.5 and 2.8) $\times 10^{-13}$ m^2/s. Likewise, it was demonstrated (Zizovic et al. 2005) that the t_d values calculated are in a good agreement with those estimated on the basis of experimental data and yield time curve (Reverchon et al. 1993) for the point of the extraction regime change. With regard to the time necessary for breakage of peltate glands (t_d), Zizovic et al. (Zizovic et al. 2005) have, initially, considered that all the glands that will undergo disruption due to supercritical fluid exposure would crack at the same time. Later, in 2008, the authors (Stamenić et al. 2008) incorporated a term of Cracking Time Distribution (CTD) in the form of Gamma function that was experimentally verified during the extraction process, into the MSMM model.

The MSMM model was developed from the secretory structure of the plant material, i.e. the location of essential oil within the plant material. Note that a specific SFE process of targeted plant matrix may not be fully understandable if not taking into account plant physiology knowledge since different plant secretory structures possess different mass transfer mechanisms. So far, four basic types of secretory structures have been identified: glandular trichomes, secretory ducts,

secretory cavities and secretory cells, and, thus, four different specific equations have been proposed to describe the whole SFE process.

It should be mentioned that the incorporation of a physical description of vegetable substrate into a mathematically theoretical model was proposed for the first time by Sovová (Sovová 1994). Later, Reverchon (Reverchon 1996) investigated the $SCCO_2$ of the essential oil from sage leaves and assumed that the oil is stored in vacuoles inside the cells and that the fraction of oil freely available on the particle surface was not significant, leading to negligible external mass-transfer coefficient. Glandular trichomes as essential oil reservoirs have firstly been incorporated into SFE mathematical modeling by Reis-Vasco et al. (Reis-Vasco et al. 2000). Assuming that part of the essential oil was stored in glandular trichomes and part—in internal structures of a leaf, the authors took into account the desorption of essential oil located near the leaf surface and the extraction mass transfer resistance regarding the part of essential oil contained in the internal part of the vegetable structure.

In 2003, Gaspar et al. (Gaspar et al. 2003) developed a model which incorporates storage of oregano essential oil in glandular trichomes and plate geometry of herbaceous particles, but gland behavior during the extraction process was not considered. In fact, due to the rapid decompression of CO_2 expansion, a disruption of glandular trichomes took place. Thus, the method for glandular trichome disruption induced by the rapid CO_2 expansion can be taken as a pretreatment for the SFE (Meyer et al. 2012b; Gaspar et al. 2001, 2003).

However, these models cannot not explain the obvious *S* shape of the extraction yield curve obtained during SFE of essential oils from *Lamiaceae* family species (Reverchon et al. 1993; Reis-Vasco et al. 2000; Goodarznia and Eikani 1998), which indicates a change in the $SCCO_2$ extraction regime. Interestingly, the slope of the extraction yield curve in the point of the regime change is similar to the slope of the curve at the beginning of the extraction process. This implies that in this stage, the rate of the extraction is almost the same as that at the beginning of the process; this indicates that at the point of regime change some new quantity of essential oil is released and becomes easily available for $SCCO_2$. To explain the origin of newly released oil quantity during extraction process, it may be assumed that a fraction of peltate glands is disrupted during extraction due to the exposure to supercritical fluid (Zizovic et al. 2005). Since the peltate gland membrane is permeable, the $SCCO_2$ can penetrate and dissolve into the oil phase. In turn, dissolved CO_2 in the oil phase will increase the peltate gland volume, thereby inducing peltate membrane stretching, and eventually membrane disruption and opening for a fraction of the peltate glands. This assumption has been experimentally verified and peltate gland disruption, due to exposure to $SCCO_2$, was confirmed by SEM analysis (Zizovic et al. 2005). Systematically, Zizovic and coworker have brought new ideas for describing the process of $SCCO_2$ extraction of essential oils and the kinetic process can be simulated by the MSMM model which observes mass transfer on the secretory-structure-scale. This approach has been applied to the process of SFE from glandular trichomes (Zizovic et al. 2005; Stamenić et al. 2008), secretory cells and cavities (Zizovic et al. 2007a; Stamenić

et al. 2008), and secretory ducts (Zizovic et al. 2007b; Stamenić et al. 2008). These works have revealed that the SFE is highly dependable on the secretory structure, i.e. its shape and location within the plant material. Based on these studies on the micro-scale and the behavior of specific secretory structure during the SFE, the classification of plant material can be made according to the model parameters values such as external mass transfer coefficient, diffusivity coefficient of the plant tissue or internal mass transfer coefficient (Stamenić et al. 2008). Accordingly, external mass transfer is dominant in the extraction from plants with secretory ducts (all the essential oil content is easy available to $SCCO_2$) and secretory cavities of citrus family (dimensions up to 0.9 mm are totally destroyed by the grinding process) while internal diffusion through the plant material is the rate limiting step for the extraction from secretory cells. In the case of peltate glands, external mass transfer and diffusion through the gland membrane will both influence the extraction process.

As suggested by the authors, the MSMM model can be used with confidence to optimize the SFE process with the view to reduce supercritical solvent consumption. The optimal SFE process should include a grinding followed by herbaceous matrix exposure to supercritical fluid as a batch (non-flow) in order to enable peltate glands disruption before the continuous flow extraction. Experimentally determined reductions of supercritical CO_2 consumption, due to rapid CO_2 decompression pretreatment (Stamenić et al. 2010; Stamenić and Zizovic 2013) in the case of SFE of essential oils from mint, rosemary, sage, valerian, ginger, mentha, hyssop and wild thyme on the laboratory scale, are in the range of 0.171–1.23 kg CO_2/g oil for the obtained oil yield of 0.80–3.25 % produced essential oil extract, and correspondingly the reductions of energy consumption are in the range of 24.9–235.0 kJ/g oil, which is strongly dependent on the yield chosen.

However, the MSMM model on the micro-scale is limited to describing the process of SFE of essential oils when the oil solubility is low. Furthermore, it is valid only for SFE from natural matters with secretory structure as essential oil reservoirs at pressures around 10 MPa when essential oil content is dominant in the extract. Indeed, the model has been successful with modeling the process of SFE of essential oils from a number of natural species (Zizovic et al. 2007b). For instance, Zizovic et al. have used this model with a very high accuracy to simulate $SCCO_2$ extraction process of basil, rosemary, marjoram and pennyroyal, resulting in an average deviation of less than 0.83 % from the experimental data (Zizovic et al. 2005). On the other hand, at higher pressures, this microscale model is not applicable because, besides essential oils coming from specific secretory structure, heavier compounds that are spread out within the plant material can be also extracted (Stamenić et al. 2010). Consequently, for the modeling of the SFE at higher pressures, the authors have turned to the most widely used BIC model in the literature for describing the process of SFE from plant materials (Stamenić et al. 2010; Meyer et al. 2012a).

3.5 Other Relatively Simple Models

Apart from the above-mentioned BIC, SC and micro-structured theoretical models, there are a number of relatively simple mathematical models frequently reported in the literatures. These models include the heat analogue diffusion model, desorption model, and logistic model addressed later. These models can be readily applied as they provide a direct solution to the overall extraction curves in terms of the extraction yield. The details for these models can be found in a review paper (Huang et al. 2012). Herewith, just brief descriptions will be given.

The heat analogue diffusion model treats the $SCCO_2$ extraction process as a heat transfer phenomenon where all solid particles are considered to be spherical and cool down in a uniform surrounding like hot balls. Then, the equations that apply to the cooling of the "hot ball" can be used to describe the concentration profile inside the particles as a function of time. Based on these assumption, Bartle et al. (Bartle et al. 1990, 1992) developed a heat analogue diffusion model or hot ball model for the extraction of 1,8-cineole from rosemary leaves and investigated the effect of matrix shape, size variation, solute distribution and solubility limitations on the hot ball model for describing real extraction systems. Unlike the BIC hypothesis (Sovová 1994), this model is suited for cases where intraparticles or internal diffusion rather than solubility-limitation controls the extraction mass transfer processes. Later on, a number of researchers have further elaborated this model for better simulating various extraction processes of natural materials (Reverchon et al. 1993; Moura et al. 2012; Čretnik et al. 2005; Macías-Sánchez et al. 2009; Rochová et al. 2008; Andrade et al. 2012; Mehr et al. 1996; Subra et al. 1998; Esquível et al. 1999; Hojnik et al. 2008; Ohira et al. 2009; Domingues et al. 2012; Almeida et al. 2013). As addressed elsewhere (Huang et al. 2012), this model performs very well with satisfactory calculations for various SFE systems, as reflected by acceptable correlation deviations for the experimental data.

The model describes the extraction process as a series of stages: $SCCO_2$ diffusion in the film around the particle followed by penetration and diffusion into the particle; compound solubilization in $SCCO_2$, and subsequent diffusion through the particle and through the SCF film. Assuming homogeneous distribution of the solute inside the particles, applying the Fick's second law of diffusion, heat–mass transfer analogy and Fourier transforms, the fraction of the solute that is extracted from the bed against time can be given by

$$\frac{m_t}{m_0} = 12\sum_{k}^{\infty} \frac{(\sin\beta_k - \beta_k \cos\beta_k)^2}{\beta_k^3(2\beta_k - \sin 2\beta_k)} \cdot \left\{1 - \exp\left[-\left(\frac{\beta_k}{R}\right)^2 D_e t\right]\right\} \tag{3.41a}$$

$$\beta_k \cot\beta_k = 1 - k_f \frac{R}{D_e} \tag{3.41b}$$

where m_t and m_0 are the total cumulative amount of the solute extracted at time t (g), and the total mass of the extractable solute loaded in the fixed bed (g), respectively.

D_e is the effective solute diffusivity in a solid substrate (m^2/s), and k_f is the external particle-to-fluid phase mass transfer coefficient (m/s), R is radius of equivalent spherical particles (m), and β_k is the k^{th} term of the series solution defined by the above periodic Eq. (3.41b).

Simply, if the solute film resistance is neglected, $k_f/D_e \to \infty$, then $\beta_k = n\pi$, thus Eq. (3.41) can be further reduced to the familiar expression with only one adjustable parameter:

$$\frac{m_t}{m_0} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\cdot\exp\left[-\left(\frac{n\pi}{R}\right)^2 D_e t\right] \tag{3.42a}$$

Equation (3.42a) can be simplified further by neglecting the higher order term for short time extraction, resulting in

$$\frac{m_t}{m_0} = \frac{6}{\sqrt{\pi}}\left(\frac{D_e t}{R^2}\right)^{1/2} \tag{3.42b}$$

On the other hand, all terms except the first in the series of exponential terms in Eq. (3.42a) may be neglected for long time extraction, and the extraction yield curve for this period can be described as:

$$\frac{m_t}{m_0} = 1 - \frac{6}{\pi^2}\exp\left(-\frac{\pi^2 D_e t}{R^2}\right) \tag{3.42c}$$

The heat analogue diffusion model can be easily applied to estimate the values of the solute diffusion coefficient D_e regardless of the fact that the resultant D_e values concerning vegetable matrices differ by several orders of magnitude and depend strongly on the system temperature, pressure, CO_2 flow rate and the solute microstructures. The D_e values are mostly in the order of (10^{-15} to 10^{-13}) m^2/s (Reverchon et al. 1993; Ohira et al. 2009); however, sometimes, they could be approximately in the order of (10^{-19} or 10^{-17}) m^2/s (Macías-Sánchez et al. 2009) as well. Macías-Sánchez et al. (Macías-Sánchez et al. 2009) explained that with the difference in the material structure. Their conclusion was based on thorough and very careful parallel comparison investigations.

However, as the extraction proceeds, the solute concentration in the bulk fluid may not be negligible, especially if a large amount of accessible solute is assumed to cover the particle surface. Then, the heat analogue diffusion model is not valid any more. Some researchers like Goto et al. (Goto et al. 1996) and Macías-Sánchez et al. (Macías-Sánchez et al. 2009) have recommended that the choice of the most appropriate mass transfer model should be made by evaluating the values of the Biot number $B_i = 2Rk_f/D_e$. Thus, if the B_i value is higher than 10 or if the values of k_f/D_e are high, then the internal diffusion can be the controlling factor and it will be more appropriate to choose the heat analogue diffusion model for simulating the extraction process instead. But once the B_i value is much less than 10 or the values

of k_f/D_e are low, then the controlling factor is probably the mass transfer in the interstitial fluid and the above-mentioned diffusion model can not be applied in such a case. For many cases, the Biot number values are very high, indicating that the internal diffusion may possibly control the extraction process. For instance, Macías-Sánchez et al. (Macías-Sánchez et al. 2009) have investigated the $SCCO_2$ extraction of carotenoids from microalgae, and found that the Biot number calculated is in the order of 10^7–10^{10}. These values are much higher than 10 and possibly useful in the scaling-up design of the extraction process. One may note that the SC model can be reduced to the equivalent heat analogue diffusion model if $K/k_f << R/5D_e$ and $K << 1$ (Catchpole et al. 1996). In other words, the rate of extraction is intraparticle diffusion controlled and the extract solubility in $SCCO_2$ is very high compared to the initial extraction concentration in the herb material.

The desorption model, proposed by Tan and Liou in 1989 (Tan and Liou 1989b), is used to describe the SFE process as desorption of the solute from the parent matrix. This model assumes the interfacial mass transfer as a first-order kinetic model and is represented by the following equation:

$$m_t = F \cdot \frac{1-\varepsilon}{\varepsilon} x_0 \rho_s \frac{1}{k_d}\left[1 - \exp\left(\frac{\varepsilon H A k_d}{F}\right)\right][\exp(-k_d t) - 1] \tag{3.43}$$

where k_d is the desorption rate constant (s^{-1}); F is volumetric flow rate of the $SCCO_2$ solvent (m^3/s); x_0 is the initial solute concentration in the solid phase (g solute/g solute free solid bed); $A(m^2)$ *and* $H(m)$ are the cross-sectional area and height of the cylindrical extraction unit, respectively.

This single parameter desorption model performed very well for describing the removal of toluene from activated carbon with by $SCCO_2$ (Tan and Liou 1989b). Thus later on it has frequently been applied to the extraction of oil from Shiitake mushroom (Kitzberger et al. 2009), oleoresin from marigold (Campos et al. 2005), and oil from carqueja (Silva et al. 2009), essential oil form Eucalyptus globulus bark (Domingues et al. 2012), curcumins from turmeric rhizomes (Chassagnez-Méndez et al. 2000), oil from pupunha (Araújo et al. 2000).

The logistic model (Martínez et al. 2003), derived from the BIC model (Sovová 1994), have been developed by neglecting the accumulation and dispersion in the fluid phase as these phenomena have no significant influence on the process while compared to the effect of convection. Following this model, the OEC can be represented by:

$$\frac{m_t}{m_0} = \frac{1}{\exp(bt_m)}\left\{\frac{1+\exp(bt_m)}{1+\exp[b(t_m - t)]} - 1\right\} \tag{3.44}$$

where $b_{lm}(s^{-1})$ and t_{lm} (s) are two adjustable characteristic parameters of the logistic model; m_t and m_0 are the total cumulative amount of the solute extracted at time t, and the total mass of the solute loaded in the fixed extraction bed, respectively.

The LM model seems to be very simple with two adjustable parameters to describe the overall extraction curves and thus it has also been applied to simulate many $SCCO_2$ extraction systems (Huang et al. 2011; Moura et al. 2012; Mezzomo et al. 2009; Kitzberger et al. 2009; Campos et al. 2005; Andrade et al. 2012; Domingues et al. 2012; Almeida et al. 2013; Martínez et al. 2003; Sousa et al. 2005).

Conclusions

Despite great advances have been achieved for simulating extraction processes of targeted species from various plant materials by means of $SCCO_2$ fluid technology, further efforts and developments are still required for the scaling-up and design of industrial commercial applications. This Chapter has attempted to present a concise review on the developments of theoretical models for supercritical fluid extraction of botanical matrices. The models examined thoroughly here include the well-known and thus extensively used broken and intact cell model, followed by the shrinking core model, microstructured mathematical model and some relatively simple models. These models generally are physically meaningful and able to describe the extraction process in terms of the overall extraction curves. The models with analytical solutions can readily be applied for correlating experimentally determined time-dependent data, but their disadvantages are also obvious as they are rather simple. Due to the complicated microstructures of botanical matters, the interactions between the solute and solid matrices may not be negligible, and for the scaling up and pilot plant design, the possible axial dispersion should be taken into account. In the meantime, microstructural properties of vegetable substrates and the effects of various pretreatments like drying, mechanical crushing, gas-aided compression or bio–processing on these properties must also be fully investigated for better understanding the $SCCO_2$ extraction results experimentally measured. In addition, some convenient and reliable optimization methods may enable us to readily work on theoretical mass transfer models for simulating the kinetics $SCCO_2$ extraction processes.

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Chapter 4
Thermophysical Properties of Pure Substances in the Context of Sustainable High Pressure Food Processes Modelling

Tiziana Fornari and Roumiana P. Stateva

4.1 Introduction

The sustainable use of renewable resources, complying with consumer health and environmental requirements, motivates the design, optimization and application of green benign processes. Particularly, concerning food industry, the increasing environmental regulations and social consciousness about the profound influence of food on health, demand to eliminate the use of petroleum-derived solvents (most of them toxic, flammable and/or corrosive). In this respect, novel technologies based on the use of high pressure solvents, open a wide range of alternatives for process and product improvement and innovation.

The application of supercritical fluids (SCFs) is a typical example of a novel technology for the ecologically compatible production and processing of natural substances. In recent years, SCF technology has considerably outgrown its first modest applications, and nowadays a substantial number of high added value substances of interest to the food, nutraceutical, pharmaceutical, cosmetic, body care and hygiene industries are produced using SCFs. Supercritical carbon dioxide ($SCCO_2$) is the most utilized SCF because of its unique properties; furthermore, CO_2 is a food-grade substance and hence its use as a solvent was very well accepted by the food industry.

The current trend in food marketing turns round the design of foodstuffs which, besides their nutritional and organoleptic value, contribute to human health and welfare. This class of foods is known as "functional foods" (Ashwell 2004) and, under the specific regulations of each country, can be labelled with attractive health

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claims. Thus, functional foods constitute a very attractive market for food industry. In Europe, sales of functional foods have increased significantly—Germany, France, UK and the Netherlands being the most important countries in the market (Jago 2009).

The advance of supercritical fluid technology in the processing of natural raw materials has joined the development of functional foods as the major allied technology. Several solid batch plants are working in the food field, such as decaffeination of green coffee beans, production of hops extracts, recovery of aromas and flavours from herbs and spices, removal of contaminants, among others. On the other side, the food processing related with liquid substances includes edible oils, by-products and wastes of the oil industry, alcoholic beverages, juices, dairy products, etc.

Another innovative technology, particularly applicable for the recovery of high value substances on a small scale production, is pressurised liquid extraction (PLE). The use of pressurized liquid solvents has permitted the increase of processing temperatures maintaining traditional organic solvents in the liquid state; thus, process selectivity is tuned and efficiency is enhanced. In the case of food-grade liquid solvents, excellent applications have been found for pressurized hot water (PHW). The dielectric constant of water is so drastically reduced by increasing temperature up to values around 200 °C that this polar solvent becomes a suitable solvent for low polar substances. The large number of novel and interesting applications developed using PHW has introduced the new expression 'subcritical water' as a special term qualifying that kind of a solvent, although, from a thermodynamic point of view, water at ambient temperature and pressure is also subcritical.

Gas expanded liquids (GXLs) and particularly CO_2 expanded liquids (CXLs) are also relatively new and promising alternative solvents, mainly because they require lower working pressures than $SCCO_2$. As CO_2 is dissolved in an organic liquid, the liquid expands volumetrically and its thermophysical properties (transport properties, polarity, solubility behaviour, acidity, etc.) might change. However, not all liquids behave in the same way. Some liquids (e.g. water) do not dissolve CO_2 significantly and therefore do not expand much; the most traditional organic solvents (e.g. ethanol) dissolve large quantities of CO_2 and therefore expand significantly; ionic liquids and some polymers are intermediate between previous classes.

In general, the compressed CO_2 in GXLs acts as an anti-solvent and can be used to induce crystallization of solutes. Particle formation is increasingly important, particularly in the production of foodstuffs, nutraceuticals and drugs. A large number of techniques have been developed, nominated using diverse acronyms such as PGSS (particles from gas-saturated solution), GAS (gas antisolvent), SEDS (solution enhanced dispersion by supercritical fluids), DELOS (depressurization of an expanded liquid organic solution), among others.

To exploit high pressure fluids to their full advantage in the processing of food substances, the phase equilibria behaviour needs to be understood. The design, optimization and development of high pressure technologies require a detailed

knowledge of the phase behaviour of the systems involved and, above all, of the solubility of solutes in the high pressure solvent. Although the solubilities of a great variety of compounds, of interest to the food industry, in $SCCO_2$, and $SCCO_2$ with cosolvents, have been determined in the last decades, still, more and more solubility data of various complex natural substances are needed. Furthermore, despite the growing number of applications of PHW, solubility measurements have been concentrated on polycyclic aromatic hydrocarbons, and data corresponding to high added value solutes within a wide range of temperature and pressure are virtually absent.

The accurate experimental determination of the solubility of compounds in high pressure fluids, however, is laborious, often difficult and time-consuming. Thus, there is a clear-cut need of reliable, robust and efficient tools capable of describing the thermodynamics of mixtures of green high pressure solvents and natural substances. These theoretical developments are necessary for engineers and academia alike in order to exploit to the full the advantages of the singular properties of high pressure fluids as solvents in the processing of high added value substances. Nevertheless, the development of such tools is a very challenging and demanding task as these mixtures can exhibit intricate phase behaviour.

The concepts, structure, and elements of a hierarchical framework targeted on modelling the thermodynamics of processes utilizing high pressure fluids will be outlined, focussing the readers' attention particularly on one of its elements, namely the thermophysical properties of pure substances, and the methods to estimate them. The reason behind that is that knowledge of pure component properties is a prerequisite for efficient, robust and reliable modelling and design of SCF processes' thermodynamics.

4.2 Thermodynamic Modelling Framework: Elements, Structure and Organisation

A thermodynamic modelling framework (TMF) comprises three main elements: a library of thermophysical properties of pure substances and methods for estimating the missing ones; thermodynamic models for mixture properties; and methods, algorithms and numerical techniques for solving the equilibrium relations. The TMF should be based on upfront scientific knowledge and incorporate the most advanced methods since it will act as a key element in the fast screening, evaluation and optimization of process alternatives and will provide quickly answers to any controversy about the production before investment decisions are made.

Building a TMF particularly focussed on SCF processes is far from trivial as a number of challenges and difficulties inherent to the modelling and prediction of the phase behaviour of the complex systems with a supercritical solvent, which are not usually encountered in other phase equilibrium calculations, should be taken into account and resolved. In particular:

1. *The vapour pressure is the most important indicator of solubility*. However, together with additional pure-component data it is often unavailable and cannot be measured experimentally for relatively non-volatile complex solids, which are most of the solutes of interest.
2. *The proximity to the critical point*. The rapid density changes and the anomalous behaviour displayed in the critical region is a challenge to any model applied near the critical point, which is mathematically singular.
3. *SCF solutions are often highly asymmetric*. The solute and solvent molecules generally differ greatly in molecular size and in their interaction strengths, leading to highly non-ideal mixtures. As a result, binary interaction constants must be correlated from data using conventional corresponding states theory based on critical properties.
4. *SCF solutions are highly compressible*. This leads to solvent condensation or clustering about the solute even in non-polar systems.

The three elements of the TMF are closely interrelated. The element on the lowest level is the library with pure component properties. It contains also a number of methods to estimate the missing properties. The latter is extremely important as the objects of the food industry are usually substances for which there are either very limited or completely missing property data. Once the missing properties are estimated, the information is sent to the element Thermodynamic models for mixture properties. The results of a thermodynamic model performance can reveal and supply important information about the quality of the thermophysical properties estimated and in some cases a different method/methods for estimation can be tried until an acceptable agreement between the available experimentally measured and the calculated phase equilibria data is achieved.

The third element of the TMF is of utmost importance as its main task is to model and predict the richness of the phase behaviour, observed experimentally in systems with a supercritical fluid. It comprises methods for thermodynamic stability analysis of complex non-ideal multicomponent systems at supercritical conditions, and reliable and robust flash routines interwoven in an appropriate software medium.

The product of a TMF is the modelling of the thermodynamics of a SCF process. Its reliability and robustness depend on the performance of each element of the TMF, on the quality of information it exchanges with the other elements and on the harmonious interrelation of all elements. A concise paradigm of the TMF organisation is shown on Fig. 4.1.

The data produced by the TMF is used to develop flowsheets of SCF installations; it is a vital element for their economic evaluation, sustainability and environmental analysis, etc. The TMF will thus save time and money, because it will advise which alternative solutions are promising and are worth investing in.

The design and development of technologies for processing high-added value food substances require that the TMF should be able to model and calculate the phase equilibria of solid and liquid materials in high pressure solvents. In view of this, we begin by a brief outline of the thermodynamics of the phase equilibria

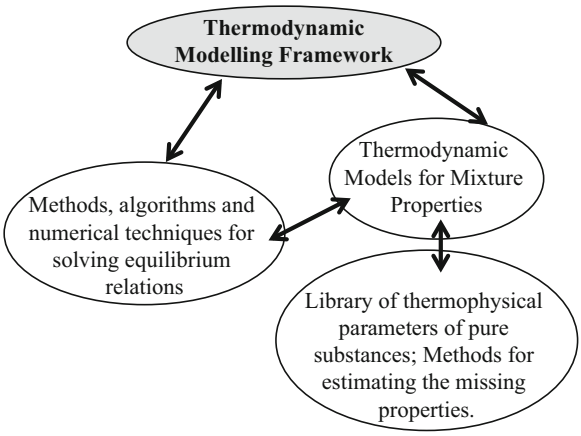

Fig. 4.1 Thermodynamic modelling framework: elements, structure and organisation

exhibited by a liquid or solid substance with a SCF solvent, and by a solid material with high pressure liquid solvent.

4.3 Thermodynamics of High Pressure Phase Equilibria

The relevant applications of SFE in the food industry target (i) the calculation of solid solubilities in $SCCO_2$ (solid + SCF phase equilibria) and (ii) the calculation of liquid + SCF equilibrium, exhibited by liquid food materials processed for example in counter current packed columns. Furthermore, with respect to subcritical water processing, the phase equilibria between a solid and hot pressurized water has to be represented as well.

4.3.1 Liquid + Supercritical Fluid

The extension of the well-known equifugacity vapour-liquid equilibrium condition to a liquid material which is in contact with a supercritical fluid (SCF) leads to:

$$f_i^{\mathrm{L}} = f_i^{\mathrm{SCF}} \tag{4.1}$$

where f_i^{L} and f_i^{SCF} are the fugacities of component i in the liquid and supercritical phases, respectively.

Fugacities are related as shown:

$$f_i^{\mathrm{L}} = x_i \varphi_i^{\mathrm{L}} P$$
$$f_i^{\mathrm{SCF}} = y_i \varphi_i^{\mathrm{SC}} P \tag{4.2}$$

where P is the pressure, x_i and y_i are mole fractions, and φ_i^{L} and φ_i^{SC} are fugacity coefficients of the i-th component in the liquid and supercritical phase, respectively.

Taking into account Eq. (4.1), the equifugacity condition can be expressed as:

$$y_i \varphi_i^{\mathrm{SC}} = x_i \varphi_i^{\mathrm{L}} \tag{4.3}$$

The calculation of the equilibrium compositions of the i-th component in the liquid and supercritical phases depends on the way its fugacity coefficients are calculated, which in its own right requires a thermodynamic model. In this respect, Cubic Equations of State (CEoS) derived from the equation first proposed by van der Waals are currently the most widely used models for representing the phase equilibria of asymmetric systems at high pressures (Fornari et al. 2010).

4.3.2 Solid + Supercritical Fluid

The majority of compounds of interest to the food industry are typically pure solids and hence there are a lot of systems which exhibit solid-supercritical fluid phase equilibria. Four different modelling approaches are used to describe the solubility of solids in SCFs: (i) density based correlations; (ii) dense gas and (iii) expanded liquid approaches and (iv) the solubility parameter method.

The density based method is an empirical or semi-empirical modelling approach which relies on developing a relationship between the solubility of a solute and the density of the SCF. The most popular model was developed by Chrastil in 1982, and is based on the solvato complex model, which establishes a linear dependency, on logarithmic basis, between the isothermal solute solubility and the SCF density. This equation has been widely employed to correlate solubility data (Güçlü-Üstündag and Temelli 2000, 2004) and has been also used to judge the goodness of experimental solubility measurements. However, few works were reported in the literature modifying or improving Chrastil's equation (del Valle and Aguilera 1988). Recently, Fornari et al. (2009) demonstrated that this type of density depended correlations can also be employed to represent the solubility of supercritical fluids in organic solvents, what can be a useful approach for the modelling of GXLs processes (Hernández et al. 2011).

The dense gas and expanded liquid approaches are both equation of state (EoS) approaches that differ in the way in which the SCF phase is treated. At high pressures and liquid like densities SCFs can be treated either as a gas or as a liquid (McHugh et al. 1988; McHugh and Krukonis 1994). The dense gas approach treats the SCF as a gas while the expanded liquid approach treats the SCF as a liquid.

The solubility parameter method is an expanded liquid approach which uses the Regular Solution Theory and the solubility parameter concept to develop a model for the solubility of solids in high pressure fluids. A separate section will be devoted to this approach as it is the preferred choice in the analysis and design of pressurized liquid extraction processes.

In what follows we will focus on the dense gas approach because it is currently the most widely used approach for representing the phase equilibria of asymmetric systems at high pressures.

The dense gas approach for modelling solute solubilities in SCFs begins with equating the fugacities of the solid and SCF phase and the standard formulation of this problem is based on the equifugacity condition for the solute; that is, assuming an EoS model for the fluid phase and denoting by the superscript "*S*" the solid solute and by the superscript "*SC*" the fluid phase:

$$f^{S}(T,P) = f^{SC}(T,P,\mathbf{y},V) \tag{4.4}$$

where f^{S} is the fugacity of the solute in the pure solid phase, f^{SC} is fugacity of the solute in the fluid phase solution, $\mathbf{y} = (y_1, y_2, \ldots, y_{Nc})^{T}$ is the vector of the fluid phase mole fractions of the N_c components, and V is the molar volume of the fluid from an EoS model. Additional relationship that must be satisfied is summation to one of the fluid phase mole fractions.

Two different approaches are popular to introduce mathematical artifices for estimating f^{S}. According to the first approach, originally proposed by McHugh et al. (1988), the solid vapour pressure is used as the reference fugacity of the solid solute. Thus, for the simple case of binary solid + SCF equilibria, and denoting the specific solid solute by subscript i, then:

$$f_i^{S} = P_i^{S}\varphi_i^{S}\exp\int_{P_i^{S}}^{P}\frac{v_i^{S}dP}{RT} \tag{4.5}$$

where P_i^{S} is the sublimation (vapour) pressure of the pure solid, φ_i^{S} is its fugacity coefficient at sublimation pressure and v_i^{S} is the molar volume of the solid, all at temperature T.

The fugacity of the solute in the supercritical phase is:

$$f_i^{SC} = y_i P\varphi_i^{SC} \tag{4.6}$$

where φ_i^{SC} is the fugacity coefficient, and y_i is the solubility (mole fraction) of the solute in the supercritical fluid.

Assuming that the solid phase is pure, the fugacity of the solute in the solid state is equal to the pure solid fugacity, and then Eq. (4.5) can be rewritten as follows:

$$f_i^{\mathrm{S}} = P_i^{\mathrm{S}} \varphi_i^{\mathrm{S}} \exp\left(\frac{v_i^{\mathrm{S}}\left(P - P_i^{\mathrm{S}}\right)}{RT}\right) \tag{4.5a}$$

Applying the thermodynamic equilibrium condition Eq. (4.4), the mole fraction of the solid component in the supercritical phase can be expressed as:

$$y_i = \frac{P_i^{\mathrm{S}}}{P} E \tag{4.7}$$

where

$$E \equiv \frac{\varphi_i^{\mathrm{S}} \exp \int_{P_i^{\mathrm{S}}}^{P} \frac{v_i^{\mathrm{S}} dP}{RT}}{\varphi_i^{\mathrm{SC}}} = \frac{\varphi_i^{\mathrm{S}} \exp\left[\frac{v_i^{\mathrm{S}}\left(P - P_i^{\mathrm{S}}\right)}{RT}\right]}{\varphi_i^{\mathrm{SC}}} \tag{4.8}$$

The so-called enhancement factor (E) contains three correction terms: φ_i^{S}, which takes into account non-ideality of the pure solute i sublimation vapour pressure, the Poynting correction, which gives the effect of pressure on the fugacity of the pure solid, and φ_i^{SC} which accounts for the non-ideality of the supercritical phase.

Of all three correction terms the last one is by far the most important as φ_i^{SC} is always far removed from unity and can produce very large enhancement factors. Furthermore, taking into consideration that the solubility in an ideal gas is $y_i = P_i^{\mathrm{S}}/P$, the large solubility enhancements in SCFs relative to an ideal gas are a results mainly of the exceptionally small values of φ_i^{SC}.

Equations (4.7)–(4.8) represent the basis for the dense gas approach for modelling solid solubilities in SCFs and show that the solute solubility is primarily a function of the solid solute pure compound physical properties, the system temperature and pressure, and the fugacity coefficient of the solid solute in the supercritical fluid, which is calculated by an EoS thermodynamic model.

The second approach, introduced originally by Kikic et al. (1997), implements the fugacity of a hypothetical subcooled liquid (SCL) phase, as the reference of the solid phase fugacity. Thus, the solid-phase fugacity function, for the pure solute i solid phase at temperature T and pressure P, is defined in terms of a hypothetical liquid-phase fugacity as a reference state, and disregarding the change in specific heat because of its negligible effect, is given as follows:

$$f_i^{S} = f_i^{SCL} \exp\left(\int_{P_i^{\mathrm{S}}}^{P} \frac{v_i^{S} - v_i^{SCL}}{RT} dP + \frac{\Delta H_{\mathrm{m}}}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right) \tag{4.9}$$

where f_i^{SCL} is the fugacity of solute i in the pure subcooled liquid state, ΔH_{m} is its enthalpy of fusion, T_{m} the corresponding melting temperature and $\Delta v_{\mathrm{m}} = v_i^{S} - v_i^{SCL}$

is the change in molar volume from the solid state to the subcooled liquid state, all taken for the solute at its triple point.

Implicitly assuming that there are no solid-solid phase transitions and provided that the solid molar volume at the subcooled liquid state (v_i^{SCL}) is weakly dependent on pressure, Eq. (4.9) can be written as follows:

$$f_i^S = f_i^{SCL} \exp\left(\frac{\left(v_i^S - v_i^{SCL}\right)}{RT}\left(P - P_i^S\right) + \frac{\Delta H_m}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right) \tag{4.9a}$$

In Eq. (4.9a) the data which are required for calculating the fugacity of the pure solid phase are: the fugacity of the pure solute in the subcooled liquid phase (calculated from a thermodynamic model, e.g. EoS); the enthalpy of fusion at the triple point (ΔH_{TP}), the triple point temperature (T_{TP}), the triple point pressure (P_{TP}) and the change in molar volume assumed to be a constant upon fusion (Δv_{TP}).

4.3.3 Solid + High Pressure Liquid

The fundamental equifugacity equilibrium condition for liquid water and a pure solid solute (i) is:

$$f_i^S = f_i^L \tag{4.10}$$

where f_i^S is the fugacity of the solute in the solid phase and f_i^L is the fugacity of the solute in the liquid water phase.

An adequate assumption is that the solid phase does not absorb water. Furthermore, if the solid phase is a pure compound, then f_i^S is the fugacity of the pure solid solute.

The solute fugacity in the liquid phase can be referred to the fugacity of the pure solute in liquid state f_i^o:

$$f_i^L = \gamma_i x_i f_i^o \tag{4.11}$$

where γ_i is the activity coefficient of the solute in the liquid phase, x_i is its molar fraction (solubility) and f_i^o is the liquid-phase standard state fugacity that typically is taken as the pure liquid fugacity at the system temperature and pure liquid vapour pressure, with the corresponding corrections for vapour phase non ideality and for the effect of total pressure.

Replacing Eq. (4.11) in Eq. (4.10), the following relation for the solubility is obtained:

$$\ln x_i = \ln\left(f_i^S / f_i^o\right) - \ln\gamma_i \tag{4.12}$$

where f_i^o stands for the pure solute in a hypothetical liquid state in cases when the mixture temperature is lower than the solute triple point temperature. Additionally, for most substances, there is little difference between the triple point temperature and the normal melting temperature. Thus, the ratio f_i^S/f_i^o can be calculated as follows:

$$\ln\left(f_i^S/f_i^o\right) = -\frac{\Delta H_m}{RT_m}\left(\frac{T_m}{T}-1\right)+\frac{\Delta C_p}{R}\left(\frac{T_m}{T}-1\right)+\frac{\Delta C_p}{R}\ln\left(\frac{T}{T_m}\right) - \int_{P_i^{sat}}^{P}\frac{v_i^L - v_i^S}{RT}dP \tag{4.13}$$

where T_m and ΔH_m are, respectively, the solute i normal melting temperature and enthalpy of fusion, and ΔC_p is the difference between its heat capacity in the liquid and solid states.

The first term on the right-hand side of Eq. (4.13) is the dominant, and the next two terms tend to cancel each other, especially if the mixture temperature and the solute melting temperature are not far apart. Additionally, the last term of Eq. (4.13), which takes into account the effect of pressure on the solute fugacity, is significant only at very high pressures since the difference between the solute molar volume in the liquid and solid states $(v_i^L - v_i^S)$ is negligible.

If the (solute i + water) mixture is assumed to be ideal, then $\gamma_i = 1$, and the solute solubility depends only on its melting properties and temperature. Taking into account that only the first term on the right-hand side of Eq. (4.13) is significant, then $\ln x_i^{id}$ can be expressed as:

$$\ln x_i^{id} = \ln\left(f_i^S/f_i^o\right) = -\frac{\Delta H_m}{RT_m}\left(\frac{T_m}{T}-1\right) \tag{4.14}$$

Further, taking into account Eq. (4.14), Eq. (4.12) can be written as follows:

$$\ln x_i = \ln x_i^{id} - \ln\gamma_i \tag{4.15}$$

Hence, calculation of the solute solubility in the aqueous phase requires estimation of its activity coefficient in the aqueous phase by applying appropriate thermodynamic models. The usual choice being models based on the group contribution approach, like the UNIFAC-based models, which take into account the energy interactions among molecules.

Furthermore, the Scatchard-Hildebrand Regular Solution theory permits the calculation of the solute activity coefficient in the case of athermal solutions

(i.e. non-polar or slightly polar systems) by means of the solubility parameter defined by:

$$\delta_i = \left[\frac{\Delta U_i}{v_i}\right]^{1/2} \tag{4.16}$$

where ΔU_i is the vaporization energy and is the v_i liquid molar volume. The most popular model for δ_i calculation is the Hansen Three Dimensional solubility parameter model.

4.4 Thermodynamic Models

Reliable thermodynamic models that are able to describe the extremely complex phase behaviour of the systems under consideration are a vital element of the TMF. An ideal thermodynamic model is a model which uses easily measured physical properties to predict phase equilibria at all conditions and is theoretically based. Still, till present no current model fits these criteria.

Cubic Equations of State: For example, the dense gas approach, which we have focussed on in Sect. 4.3.2, requires the evaluation of fugacities or fugacity coefficients over the entire density range studied, and hence requires an adequate relationship to describe the *P-V-T* behaviour of the supercritical fluid phase. Currently, cubic EoSs in combination with different mixing rules are the most widely used models for the calculation of solubilities of solutes in SCFs applying the dense gas approach because the interactions are too involved to justify the use of a more fundamentally based equation. In general, cubic EoSs are exceedingly simple and have been remarkably successful in modelling SCF-phase behaviour. The most widely used cubic EoSs are the Soave-Redlich-Kwong (SRK) and Peng-Robinson (PR) equations.

If the SRK cubic EoS is written in terms of compressibility Z:

$$Z^3 - Z^2 + \left(A - B - B^2\right)Z - AB = 0 \tag{4.17}$$

then the expression for the fugacity coefficient of the i-th component in an N_c component system is:

$$\ln\varphi_i = -\ln\left(Z - \frac{Pb}{RT}\right) + (Z-1)\frac{b_i}{b} - \frac{a}{bRT}\left[\frac{1}{a}\left(2a_i^{0.5}\sum_{j=1}^{N} x_j a_j^{0.5}\left(1 - k_{ij}\right)\right) - \frac{b_i}{b}\right]\ln\left(1 + \frac{b}{V}\right) \tag{4.18}$$

where:

$$b_i = 0.08664\frac{RT_{ci}}{P_{ci}} \quad \text{and} \quad b = \sum_i \sum_J x_i x_j \left(\frac{b_i + b_j}{2}\right)\left(1 - l_{ij}\right) \tag{4.19}$$

$$a_i = a_{ci}\alpha_i, a_{ci} = 0.42748\frac{\left(RT_{ci}\right)^2}{P_{ci}}, \alpha_i^{0.5} = 1 + m_i\left(1 - T_{ri}^{0.5}\right) \tag{4.20}$$

$$m_i = 0.48 + 1.574\omega_i - 0.176\omega_i^2 \quad \text{and} \quad a = \sum_{i=1}^{N}\sum_{j=1}^{N} x_i x_j \left(a_i a_j\right)^{0.5}\left(1 - k_{ij}\right) \tag{4.21}$$

$$A = \frac{aP}{\left(RT\right)^2} \quad \text{and} \quad B = \frac{bP}{RT} \tag{4.22}$$

Thus, in order to calculate the fugacity coefficient, φ_i^{SC}, which accounts for the non-ideality of the supercritical phase in Eq. (4.8), the following properties must be known for each of the pure components in the system under consideration: the critical temperature and pressure and the acentric factor.

Cubic EoS Combined with G^E Models. All of the cubic EoSs were originally developed to characterize hydrocarbons. Furthermore, the rapid density changes and the anomalous behaviour displayed in the critical region are a further challenge for EoS models. In view of the above and in order to realise an efficient application of cubic EoSs to correlating the solubility of solutes in SCFs two avenues are usually pursued—improvement of mixing rules and improvement of the existing EoS and development of new models.

For example, an alternative to van der Waals one-fluid mixing rules, and a very attractive route to developing better mixing rules, is to incorporate into the EoSs activity coefficient models. The resulting thermodynamic models combine the advantages of successful cubic EoSs and G^{E} models, like UNIFAC and demonstrate enhanced performance. Representatives of this new group of thermodynamic models are MHV2, PSRK, PR-LCVM-UNIFAC, to name just a few, and they are often referred to as completely predictive.

For example, the expression for the mixing rule in MHV2 is:

$$q_1\left(\alpha - \sum_{i=1}^{N} z_i\alpha_i\right) + q_2\left(\alpha^2 - \sum_{i=1}^{N} z_i\alpha_i^2\right) = \frac{g^{\mathrm{E}*}}{RT} + \sum_{i=1}^{N} z_i \ln\left(\frac{b_{\mathrm{mix}}}{b_i}\right) \tag{4.23}$$

where $g^{\mathrm{E}*}$ is an independently prescribed expression for the excess Gibbs energy, and q_1 and q_2 are constants.

In the above equation, the dimensionless parameters $\alpha = \frac{a}{bRT}$ and $\alpha_i = \frac{a_i}{b_i RT}$ are related to the mixture and pure compound parameters, respectively.

The expression for the fugacity coefficient is:

$$\ln\phi_i = \ln\left[\frac{RT}{P(v-b)}\right] + \left[\frac{1}{v-b} - \frac{\alpha}{v+b}\right]b_i - \ln\left(\frac{v+b}{v}\right)\left[\frac{\partial(n\alpha)}{\partial n_i}\right]_{T,n_j} \tag{4.24}$$

where the composition derivative $\left[\frac{\partial(n\alpha)}{\partial n_i}\right]$ can be calculated from the MHV2 mixing rule, using:

$$(q_1 + 2\alpha q_2)\frac{\partial(n\alpha)}{\partial n_i} = q_1\alpha_i + q_2\left(\alpha^2 + \alpha_i^2\right) + \ln\gamma_i + \ln\frac{b}{b_i} + \frac{b_i}{b} - 1 \tag{4.25}$$

In Eq. (4.24) b_i and b are calculated according to Eq. (4.19) and $\ln\gamma_i$ from the UNIFAC model.

Thus, to calculate the fugacity coefficient according to Eqs. (4.24) and (4.25) for each of the pure components in the system under consideration the following information must be available: the component's critical temperature and pressure, the R and Q parameters for each of the components' constituent groups and the group-group interaction parameters.

Other Models: Critical properties and acentric factor are the pure component parameters commonly required by thermodynamic models such as CEoSs to represent high pressure equilibria. In addition to those, however, certain models may require particular pure component parameters, usually employed to thoroughly adjust the volatility of the pure compounds. For example, in the GC-EoS model, the fugacity coefficient of a given component is calculated from the residual Helmholtz energy expression, which is calculated as the sum of two contributions: a repulsive or free-volume term and an attractive term. Pure component parameters are only required to calculate the repulsive term. The GC-EoS attractive term is a group-contribution version of the NRTL model, and has pure-group parameters and binary interaction parameters which are adjusted using binary phase equilibria data.

The GC-EoS repulsive term is modelled assuming hard sphere behaviour for the molecules, and each substance is characterized by a hard sphere diameter (d_i). The latter is calculated according to the following generalized expression:

$$d_i = 1.065655\, d_{ci}\{1 - 0.12\exp[1 - 2T_{ci}/(3T)]\} \tag{4.26}$$

where d_{ci} is the critical hard sphere diameter, given by:

$$d_{ci} = (0.08943RT_{ci}/P_{ci})^{1/3} \tag{4.27}$$

The application of GC-EoS model to calculate the fugacity coefficient of component i requires its critical temperature and pressure. Nevertheless, in order to attain a very precise representation of the vapour pressure curve of the pure compound, d_{ci} is usually fitted to a single pure component vapour pressure data (Skjold-Jørgensen 1984), usually the normal boiling point.

In order to overcome the use of empirical corrections to cubic EoSs or G^E mixing rules, the breakthrough in the modelling of polar and highly non-ideal systems came with the development of more rigorous explicit association models. Such example is the SAFT equation, a semi-empirical EoS that was developed in the late 1980s and has gained considerable popularity in both the academic and industrial communities. SAFT is derived from the first order thermodynamic perturbation theory of Wertheim (see for example Wertheim 1984a, b, 1986a, b) where the reference fluid is a hard sphere and the perturbation consists of the relatively weak dispersive attractions.

Although the approach is very successful in modelling the phase equilibria of complex systems, research dealing with its application to the modelling of the solubility of solids in SCFs is rather scarce. For example, on the basis of a limited number of solid-SCF systems it has been demonstrated that SAFT gives a slightly better correlative accuracy than cubic EoSs, while being considerably more computationally demanding (Yang and Zhong 2005). Yet, it must be underlined that a completely different conclusion could be made when the performance of cubic EoSs is compared with the SAFT/PC-SAFT for mixtures containing strongly polar or hydrogen-bonding compounds, for which the latter thermodynamic models perform substantially better than cubic EoSs.

4.5 Thermophysical Properties of High Added Value Food Substances

4.5.1 Data Bases of Thermophysical Properties

Thermophysical properties so as chemical and molecular information (chemical formulas, ionization, spectra) of pure compounds are needed in many fields of science and engineering. The relevant information must be available from the very beginning of scientific R&D and engineering applications, because it is an essential part in the synthesis, design, simulation, debottlenecking, control and optimization of any process and may affect the reliability and consistency of the entire project.

The design, simulation and optimization of high pressure processes for the development of new food ingredients, such as flavours, aromas, antioxidants, colorants, etc., nutraceuticals, food supplements or complements are no exception and require information about the thermophysical properties of pure compounds, which are present in natural feedstocks and usually are rather complex in their molecular structure.

At present, several databases that provide molecular information and experimental property data of pure chemical compounds for industrial applications are available. Although the number of organic and inorganic chemical substances registered in CAS (https://www.cas.org/) is more than 80 million, the property data that can be found in the databases are for about only 30,000–40,000

compounds. Furthermore, from these 30,000–40,000 pure substances, there are compounds which have only one or two property values associated with them. Actually, a large number of pure compounds are not included in databases due to the complexity of experimental methods for determination of thermophysical properties, which are sometimes time consuming, expensive and inaccurate. Moreover, in some cases, the thermophysical properties required cannot be measured. A typical example is the impossibility to experimentally determine the critical parameters of high molar mass triglycerides, as they decompose before reaching the theoretical critical state. Furthermore, values in databases are often an unidentifiable mixture of experimental and predicted data, and the reliability of predicted data provided might prove unreliable as demonstrated by Yan et al. (2003). In this respect, theoretical developments leading to robust, efficient and reliable correlations to estimate thermophysical properties of pure substances and mixtures are continuously a target of science.

The most renamed thermophysical chemical database of pure component properties include DECHEMA (Society for Chemical Engineering and Biotechnology) within Germany in Europe, and the DIPPR (Design Institute for Physical Properties) in the United States. Other efforts around the world include the Physical Property Data Service (PPDS) of the Institution of Chemical Engineers (United Kingdom), the program of the Engineering Sciences Data Unit (ESDU) supported by British technical organizations, the UHDE physical property program marketed by DECHEMA, the German Chemical Engineering Association (Germany), the JUSE-AESOPP physical properties program from the Japanese Union of Scientists and Engineers (Japan), and the program of the CODATA Task Group on Data for Industrial Chemicals (International Council of Scientific Unions).

DETHERM (Thermophysical Properties of Pure Substances & Mixtures) is one of the world's largest thermophysical database of DECHEMA, which provides thermophysical property data for about 38,850 pure compounds and 129,500 mixtures. DETHERM contains literature values, together with bibliographical information, descriptors and abstracts. The complete database consists of sets of property orientated packages, which are maintained and produced by external experts. This guarantees high quality and checked data. An example for such packages is the DDB (Dortmunder Datenbank) from the Dortmund Data Bank Software & Separation Technology GmbH (DDBST GmbH).

DDB was started in 1973 in the research group of Prof. Dr. J. Gmehling at the University of Dortmund, with the compilation of vapour-liquid equilibria data for normal boiling organic compounds. Later was extended to cover also pure component properties (DDB-PCP), liquid-liquid equilibrium data, excess enthalpies, activity coefficients at infinite dilution, among other thermo-physical-chemical properties.

DDB-PCP was an advanced step to supply the industry with the required experimental data, reliable predictive methods, reliable correlation parameters and modern software for the correlation and estimation of thermophysical data. DDB-PURE is now the largest data bank in the DDB, and is intensively used for the development of property estimation methods, permits the detection of errors in both

literature and data input and thus, resulted in a data collection of remarkable reliability.

On the other hand, the backgrounds of DIPPR database dates to 1960s–1970s when several organizations in the Unites States, including government agencies, such as the National Bureau of Standards (later the National Institute for Standards and Technology, NIST), industry associations, including the American Petroleum Institute (API), and universities, such as the Massachusetts Institute of Technology (MIT), initiated projects and cooperative efforts in order to develop an important thermo-physical-chemical database. In 1975, Prof. Bob Reid organized the AIChE Annual Meeting in Los Angeles to determine the level of interest in a cooperative data effort. Over 30 people attended, and the "Project Evergreen" was born with the target of creating a database with the best available data and estimates, and made "evergreen" by continually updating it as better data and methods were found. Currently, the Design Institute for Physical Properties (DIPPR) is a technical society of the American Institute of Chemical Engineers (AIChE) and has become the premier cooperative physical property data effort in the United States to satisfy process engineering needs.

The DIPPR 801 is a numerical database with a complete matrix of recommended, consistent thermophysical property data for pure chemicals of industrial importance. All available data are assessed for accuracy, thermodynamic consistency, and reliability within chemical classes. At present, DIPPR 801 contains values for 49 properties (34 constants and 15 temperature dependent properties) for each of the 2,036 pure industrially important components comprising the database. When no experimental data are available, DIPPR 801 provides established and well-evaluated methods to predict the property value. Furthermore, DIPPR 801 database has a convenient software interface which permits the prediction of properties using an automated package, the search and comparison of multiple components, view multiple components at a time, graph and plot thermophysical data points, calculate and compare properties at different temperatures, view 2-D and 3-D models of database compounds, etc.

DETHERM/DECHEMA as well as DIPPR/AIChE produce and publish thermophysical property data since decades. Both organizations share the ambition to disseminate the best in class thermophysical property data to users in industry and research. Therefore the DIPPR-801 database is now distributed by the DECHEMA within Europe. Furthermore, DIPPR is now a licensed distributor of the DETHERM database in the Americas.

4.5.2 Estimation Procedures and Predictive Approaches

As correctly pointed out by Barley et al. (2013), estimation methods for any property have to be based upon available experimental data. Still, the vast majority of the data for properties such as for example critical properties and liquid densities have been collected for the chemical industry and for structurally simple

compounds (particularly hydrocarbons) and relatively little data are available for complex multifunctional compounds.

The high-added value substances that are of interest to the food industry are medium- and large-sized solutes at ambient or slightly elevated temperatures where they are typically pure solids. Such substances usually have the following characteristics: molar mass greater than 100 g/mol; polyfunctionality (two or more functional groups); multiple molecular conformations; complex molecular interactions, such as large dipoles, high polarizability, hydrogen bonding, and charge-transfer complexes. Experimental values of thermophysical properties for these compounds are frequently not at hand and the measurements are often expensive; more importantly, as mentioned previously, the measurement of those properties cannot be realized experimentally as the compounds either decompose or polymerise before reaching their normal boiling temperature and/or critical state.

There are many methods advocated in the literature for estimating thermophysical properties, and they can be very roughly grouped as follows: (i) methods based on numerical descriptors of chemical structure; (ii) traditional QSPR models and (iii) group contribution methods. Their advantages and shortcomings, applicability, etc., have been discussed in detail in the literature, and in several resent excellent research papers and reviews, see for example Yan et al. (2003), Sola et al. (2008), Quintero et al. (2012), to name just a few.

For example, the term QSPR (quantitative structure–property relationships) is widely used for some empirical models, which are designed to find relationships/correlations between structure and properties of molecules. These relationships relate the numerical presentation of chemical structures to different physicochemical properties.

For a given property, a QSPR can be schematically represented by the following equation:

$$y_p = f\left(x_{s1}, x_{s2} \ldots x_{sk}; x_{p1}, x_{p2} \ldots x_{pm}; \beta_0, \beta_1 \ldots \beta_n\right) \tag{4.28}$$

where y_p is the property (e.g., boiling temperature, melting temperature, critical temperature) to be predicted; x_{s1}, x_{s2},..., x_{sk} are descriptors representing numerically the molecular structure of the compounds in the database; x_{p1}, x_{p2},..., x_{pm} are known property data of the compounds in the database; and β_0, β_1,..., β_n are the QSPR regression parameters (Brauner et al. 2008).

Obviously, the development of a QSPR requires a database of molecular descriptors presenting numerically the molecular structures used and a database with experimental data for a certain amount of molecules. Several approaches are available for numerical description of molecular structures and how to relate them to properties.

The group/bond contribution methods are the oldest and still very much the favourite tool of researchers for the estimation of properties from molecular structure. Since an infinite number of chemical compounds exist but there is only a limited number of functional groups, it was convenient to estimate functional

group parameters from existing data, and then predict the properties of new compounds.

Thus, the idea behind these methods is to decompose the molecular structure into particular groups and to count atoms in those groups (atom counts). Characteristic incremental values are assigned to the groups by regression of known data for the particular property. The whole molecule is then "restored" by summation of the contributions of all groups, and the property is determined as a function of the summations (Poling et al. 2004).

As discussed by Jiao et al. (2014) group contribution methods can fall into several types: (i) multi-level group contribution method, (ii) group-interaction contribution method, (iii) position group contribution method, (iv) neural network group contribution method, (v) and the combination of these methods.

The simple first-level group contribution methods such as Joback and Reid (1987), Ambrose (1978), Lydersen (1955) are the most widely used. The reason why those methods have become so popular is that they possess inherent advantages—explicit and comprehensive description of the molecules, straightforward restoration of the whole structure of the molecule, and simple calculation of the desired property. Conversely, as pointed out by Cholakov et al. (1999) and Wakeham et al. (2002), the earlier group contribution models using atom counts do not take into consideration the placement of a respective group in a molecule and hence cannot distinguish among isomers; as a result they tend to produce unreliable results for compounds which are not similar to those used in their derivation. Different avenues were pursued in an attempt to achieve greater accuracy: the number of groups has been increased; more sophisticated groups have been introduced, and more complicated rules for the restoration of the molecule from the groups have been tried.

For example, Constantinou et al. (1994) proposed a complex estimation technique based on conjugated forms. Their work was followed by a less complex method (Constantinou and Gani 1994), wherein the estimation of the compounds can be performed at two levels. Higher order groups which account for some isomeric and cyclic structures and, thereby, increase the prediction accuracy have been proposed by Marrero and Gani (2001). The increased number of groups, however, requires more experimental data in order to avoid chance correlations, as discussed in Yan et al. (2003). Dalmazzone et al. (2006) suggested a new method using Benson's second order group to predict the critical temperature and enthalpies of vaporization of covalent compounds. To some extent, these two-level group contribution methods are applicable to isomers except for the complex and polyfunctional compounds. The method of Fontdevila and Rubio which considered molecules as aggregates of the group interaction instead of the structural groups was later extended by Morejon and Fontdevila (1999) to the estimation of boiling point and critical properties.

A position group contribution method, advocated by Wang et al. (2008) uses the contribution of groups as well as the position correction. Thus, boiling and melting points, critical properties of isomers can be distinguished and estimated. A new method, applying neural networks to develop group contribution correlations was

recently proposed by Gharagheizi et al. (2008), and was later applied to predict physical properties (normal boiling temperature, critical properties, acentric factor, etc.)—see for example Gharagheizi et al. (2013).

An alternative to the group contribution methods, for predicting pure component properties, is to determine—from a vast database—a combination of molecular descriptors that defines the most "significant common features" of the described molecules (Shacham et al. 2004; Wakeham et al. 2002). The different molecular descriptors in the database may be groups and/or bonds, which are computed by procedures such as simulated molecular mechanics, quantum chemical methods, and topology of the molecules. The descriptors that are significant for the prediction of a particular constant and their weighting factors are usually found by stepwise regression techniques. These methods thus use step-wise multi linear regression (MLR) to find from a huge number of descriptors (up to 2,000) a small combination (e.g., 8), reflecting the most significant common structural features of the targeted molecules. Since the parameters of the model are determined from a relatively huge number of measured values chance correlations are limited. Furthermore, partial correlations between descriptors are reduced by not selecting pairs correlated above a certain level.

It has been demonstrated in the past, that the values of thermophysical properties of common substances have a significant effect on the modelling and design of distillation and reaction units influencing directly the design parameters and performance of equipment (Wakeham et al. 2001). This is even more true for processes like supercritical fluid extraction and processing of complex natural substances of interest to the food industry. It is thus obvious, that it is of major importance to have reliable methods to estimate the values of their thermophysical properties, because inaccuracy in the properties will influence in a major way the quality of phase equilibria prediction and correlation, which in its own right, will have a major impact on the modelling and design of the corresponding processes. Those are truly challenging tasks, moreover that for complex substances there are very big deviations between the values estimated and reported by the different authors applying different methods. In view of this a range of effective methods to estimate the solutes properties should be interwoven in the corresponding element of the TMF and available to potential users.

Lastly, it must be underlined that depending on the type of phase equilibria exhibited (please see Sect. 4.3) different thermodynamic models are applied to model, predict and correlate the solubility of the target compounds of interest to the food industry in the high pressure solvent. In each case the level of interaction between the three elements of the TMF is of different complexity.

For example, in the case of applying the dense gas approach to correlate the solubility of a solid solute in a supercritical solvent, then the pure component properties required are the critical temperature and pressure, and the acentric factor, as well as solid molar volume and sublimation pressure (please see Eqs. (4.8) and (4.18)).

If the dense gas approach is implemented according to Eq. (4.9a), then a different set of melting properties of the solid solute is required, namely its

ΔH_{TP}, T_{TP}, P_{TP} and Δv_{TP}, in addition to the critical parameters and vapour pressure. Obviously, the level of interaction between the element "Properties" and the other two elements of the TMF is more complex in this case, particularly if experimental data for some or all of the properties required are not available and have to be estimated.

4.5.2.1 Normal Boiling Point

The normal boiling point (NBP) is one of the few physico-chemical properties available for many organic compounds due to the simplicity of the measurements.

The NBP is an indicator of the strength of the intermolecular forces which bind the molecules of a compound together. The stronger the intermolecular forces, the more densely packed the atoms and, therefore, the higher the normal boiling point. Therefore, the NBP can be directly correlated to the chemical structure of the molecule. As discussed by Gharagheizi et al. (2013), having an accurate knowledge of the NBP is of great importance as it is widely applied to predict a number of key physical and physicochemical properties, which include for example critical temperature, enthalpy of vaporization, etc.

In many cases, however, experimental determination of the NBP of complex high molar mass compounds is not possible; hence predictive models based on molecular structures should be available. Reviews of some of the most important methods to estimate NBP can be found in Poling et al. (2004) as well as in several recent contributions (see for example Gharagheizi et al. (2013)) and we will not pursue this subject further.

4.5.2.2 Critical Parameters

Currently, very few correlations of thermodynamic properties used to determine the properties of a mixture contain their actual parameters. The critical constants of pure substances are used instead, based on which the physicochemical characteristics of complex fluids are calculated using such variables as temperature, pressure, and composition.

Critical constants are important not by themselves but as parameters included in the equation for determining other thermodynamic properties of substances. If there is lack of accuracy in their values for phase transitions, as an example, inconsistent equations may be obtained, up to the emergence of relationships resulting in violation of the laws of thermodynamics. For most of the solutes of interest to the food industry the critical properties may not be experimentally accessible because of thermal cracking below their critical temperature. Hence, the values of the critical parameters and normal boiling temperatures should be considered as hypothetical quantities rather than properties with any physical meaning, which have to be estimated either by correlations or group contribution methods.

Furthermore, as pointed out in the recent review of Sovová and Stateva (2011), the most widely used methods for correlating critical parameters are included as options in commercial process simulators. In view of this, and to avoid repetition, we will not present any of them here, but will just enumerate some of the methods which have been most extensively used by the researchers in the field to predict critical properties—e.g. the group contribution methods of Lydersen (1955), Joback (1984), Somayajulu (1989), Ambrose (1980), Ambrose and Young (1995), Ambrose and Tsonopoulos (1995) as well as those advocated by Constantinou and Gani (1994), Marrero and Gani (2001), etc. Interested readers can find detailed discussion and recommendations for critical properties estimation in, for example, Poling et al. (2004); while the recent review of Fornari et al. (2010) presents analysis focused on the methods applied to estimate the critical parameters of solid solutes of interest to the food industry.

At this stage we feel that potential users should be cautioned regarding the acceptance and application of estimated data, particularly when there are no experimental data available with which to compare the estimated values. In some cases, the values of the critical parameters estimated by different methods can differ considerably, and hence an assessment should be made beforehand.

In view of this, it is recommendable to apply as an assessment tool of the reliability of the properties estimated the generalized semi-theoretical expression advocated by Zbogar et al. (2006):

$$T_c/P_c = 9.0673 + 0.43309\left(Q_W^{1.3} + Q_W^{1.95}\right) \tag{4.29}$$

where T_c is in Kelvin and P_c is in bar. The dimensionless parameter Q_w is a measure of the van der Waals molecular surface area and is calculated as the sum of the group area parameters, Q_k:

$$Q_w = \sum_k \nu_k Q_k \tag{4.30}$$

where ν_k is the number of times group k appears in the molecule. The group area parameters Q_k are available in the UNIFAC tables.

4.5.2.3 Density

Modern computer simulation packages for design often require the normal boiling point and a standard relative liquid density (d_4^{20}) of the pure components of a mixture that occur in a process (Wakeham et al. 2002). Liquid density is a fundamental characteristic of a fluid by itself, and as such is often required as the input from which other physical properties can be estimated.

Group contribution models of different levels of description of the molecular structure are the most common tool employed for the estimation of liquid density. Still, as pointed out by Wakeham et al. (2002), correlations which ensure a

compromise between reasonably high precision of the estimated properties but do not involve unduly complex descriptors and rules, should be developed.

A comparison of liquid density estimation methods limited to a set of 35 compounds can be found in Poling et al. (2004), and in a recent critical assessment of methods for estimating density of multifunctional organic compounds (Barley et al. 2013). On the basis of their work, Barley et al. (2013) recommend a scenario for estimation of liquid densities of multifunctional compounds: firstly to estimate the molar volume at T_b using the Schroeder group contribution method (Barley et al. 2013) and then correct to the experimental temperature using the Rackett equation (1970) with critical properties by either Nannoolal et al. (2007) or Joback (Joback and Reid 1987). Interested readers are prompted to examine in more detail the above contributions.

4.5.2.4 Acentric Factor

The acentric factor is a parameter that was originally defined by Pitzer (1955) to improve the accuracy of corresponding state correlations for heavier and more complex compounds. There are many procedures advocated to estimate unknown acentric factor. For example, the Edmister correlation (1958), Lee and Kesler estimation technique (1975), to name just a few, are an integral part of any process simulator. However, the accuracy of these methods mainly depends on the accuracy of the input parameters (normal boiling point, the critical pressure, and critical temperature).

It is possible to directly estimate the acentric factor via group/bond/atom contribution methods. A brief enumeration of some of those methods is given in a recent contribution of Wang et al. (2012) and will not be discussed here. Suffice it to say that Wang et al. (2012), on the basis of their universal positional distributive contribution method for the prediction of properties of organic compounds, such as critical properties, normal boiling point, and enthalpy of vaporization (Wang et al. 2008, 2009), advocated an extension for the prediction of the acentric factor of a variety of pure organic compounds. The authors demonstrate a very favourable comparison between experimental and calculated data for acentric factor applying the new method.

4.5.2.5 Melting Properties

The melting properties required by a thermodynamic model to correlate the solubility of a solid solute (as discussed Sect. 4.3) are its melting temperature, solid molar volume, sublimation pressure, triple point temperature (T_{TP}) and pressure (P_{TP}) and enthalpy of fusion at the triple point (ΔH_{TP}). In what follows a discussion of the methods available for estimation of the above properties is presented briefly:

Normal Melting Temperature. Even though the melting temperature of a pure substance can be measured accurately, its prediction has been a notoriously difficult

problem (Hughes et al. 2008). Along those lines, in several recent contributions (e.g. Preiss et al. 2011; Brauner and Shacham 2013) was commented that the difficulties to develop methods for prediction of melting properties of pure compounds are a result of numerous factors (e.g. ionic, polar and hydrogen bonding forces, crystal packing, and positional, expansional, rotational, and conformational entropy effects, etc.), which affect the solid state properties, but have very little or no effect on the liquid or gas phase properties. As a result property prediction techniques are significantly less reliable when applied to solid properties compared to their reliability in predicting liquid and gas phase properties.

The methods advocated to estimate melting points are based on either group contribution methods or physical and structural molecular parameters such as molecular cohesiveness, bulkiness, hydrogen-bonding parameters, and geometric factors, see for example Dearden and Rahman (1988), Abramowitz and Yalkowsky (1990), Dearden (2003), Godavarthy et al. (2006), to name just a few.

In 2004 Jain et al. introduced a combination of additive group contributions and non-additive molecular parameters to estimate the normal melting points of 1,215 organic compounds. The melting points are thus calculated from the ratio of the total phase change enthalpy and entropy of melting. The total phase change enthalpy of melting is calculated from the enthalpic group contributions, whereas the total phase change entropy of melting is estimated using a semiempirical equation based on only two nonadditive molecular parameters.

As pointed out by Brauner and Shacham (2013), the most widely used methods for predicting T_m are by far the group contribution methods, some of which are already implemented into commercial software packages (e.g., the Dorthmund Data Bank, DDBST, 2011 release, http://www.ddbst.de, and CRANIUM, Molecular Knowledge systems http://www.molecularknowledge.com/).

It has been demonstrated that the prediction errors resulting when applying group contribution methods to estimate T_m are much higher than the ones obtained for predicting, for example, normal boiling temperature (Brauner and Shacham 2013). In view of this, more complex methods, aiming at improving the accuracy of T_m estimations, have been advocated. For example, Godavarthy et al. (2006) advocated a QSPR method, which uses 16 molecular descriptors in a nonlinear model whose parameters were determined using a neural network; Lazzus (2009) suggested the use of a neural network and a particle swarm algorithm to represent the nonlinear relationship between the contribution of the various groups to T_m. These more sophisticated methods reduced somewhat the average prediction errors; still, the errors are considerably higher than the theoretical limit (Brauner and Shacham 2013).

Finally, a simple and fast method to calculate melting point of a substance from its boiling point was originally developed by Gold and Ogle (1969) and suggested later by Lyman (1985):

$$T_m = 0.5839 T_b \tag{4.31}$$

Sublimation Pressure, Enthalpy and Entropy of Melting (Fusion)

Sublimation pressure. It has been demonstrated that the sublimation pressure plays a dominant role in the correlation of solubility data and that, in many cases, the only way to obtain a reasonable calculation of these data is to consider the sublimation pressure as an adjustable parameter (Reverchon et al. 1995; Neau et al. 1996). It has also been shown that, in the case of high molar mass compounds for which sublimation pressures cannot be measured, the safest way to estimate them is to correlate experimental vapour pressure data through an analytical relation and to use normal fusion properties in order to settle the sublimation pressure equation with respect to temperature (Neau et al. 1999). Interested readers are prompted to the works of Neau et al. (1999) and Crampon et al. (2004) who discuss in detail the reliable methods, among the numerous ones, devoted to the estimation of vapour pressure.

Thus, a possible route to estimate the sublimation pressure of a solid compound is to integrate the Clapeyron relation from the triple point temperature T_t and pressure P_t, assuming a negligible dependence of the sublimation enthalpy with respect to temperature (Neau et al. 1999):

$$\ln\left(\frac{P^S}{P_t}\right) = -\frac{\Delta H^S}{R}\left(\frac{1}{T} - \frac{1}{T_t}\right) \tag{4.32}$$

where ΔH^S is the sublimation enthalpy at the triple point of the pure component, which can be expressed with respect to the fusion and vaporization enthalpies as:

$$\Delta H^S = \Delta H^{fus} + \Delta H^{vap} \tag{4.33}$$

The practical interest of this method is thus to require, besides an EoS, fusion property data which can be either measured, found in the literature, or estimated (Garnier et al. 1999).

In most cases the triple point conditions (the temperature and pressure at the triple point) for the solute are unknown experimentally. However, for almost all heavy compounds there is little difference between the triple-point temperature and the normal melting temperature. Indeed, this difference is usually less than 0.1 K, which is less than the scattering of experimental values of transition temperatures found in the literature. Furthermore, the difference in the heats of fusion at these two temperatures is also often negligible (Perry and Green 1999). Under this assumption, the enthalpy of fusion in Eq. (4.33) can be estimated with the fusion enthalpy measured at the normal melting temperature. Hence, if the melting temperature of the compound of interest is known it is possible to calculate P_t and ΔH^{vap} from an EoS.

An interesting approach to calculate the sublimation pressures of pure solids from high-pressure solubility data using genetic algorithms is advocated by Valderrama and Zavaleta (2005). The sublimation pressure is considered as a

parameter to be determined by regression analysis of experimental solubility data, and hence an optimization problem, in which the difference between the correlated and experimental data of solubility is to be minimized, is solved using a method, which applies biologically derived techniques such as inheritance, mutation, natural selection, and recombination to find the optimum solution.

Another route to pursue is presented by Coutsikos et al. (2003) who introduce a group-contribution method for the prediction of the vapour pressures of organic solids, based on the concept of the hypothetical liquid:

$$\ln P^{\mathrm{S}} = \ln P^{\mathrm{L}} + \frac{\Delta S^{\mathrm{fus}}}{R}\left(1 - \frac{T_{\mathrm{m}}}{T}\right) \tag{4.34}$$

The vapour pressure of the hypothetical liquid is obtained using the Abrams–Massaldi–Prausnitz equation developed for liquids (Abrams et al. 1974), and the entropy of fusion is estimated applying a group-contribution method. Coutsikos et al. (2003) underline that experimental values for the entropy of fusion for most of the compounds are not available and reliable predictions of these values are extremely difficult and the few available correlations are cumbersome to use (e.g. Chickos et al. 1990, 1991; Dannenfelser and Yalkowsky 1996). Thus, the authors advocate a group-contribution method to predict values for the entropy of fusion with typical deviations from the experimental ones—where available—of about (± 10 to ± 25) %. Taking into consideration the simplicity of the proposed method for such a difficult task, it appears sufficient for the needs for sublimation pressure predictions. The overall error for the families of organic compounds considered (hydrocarbons, halogenated aromatics, nitro-aromatics, alkanols, phenols, acids, ketones and multifunctional ones) is in the range of (10–40) %, and the error for the total of about 2,650 data points exceeds one order of magnitude in very few cases.

As pointed out above, in the case of compounds which may decompose before the melting point, all fusion properties have to be estimated.

Enthalpy of Melting. The enthalpy of melting is based on the sum of intermolecular interactions and can be therefore estimated by additive group contributions (Jain and Yalkowsky 2006).

$$\Delta H_m = \sum n_i m_i + \sum n_j m_j \tag{4.35}$$

where n_{i} is the number of times that group i appears in a compound, n_{j} is the number of times that proximity factor j appears in a compound, m_i is the contribution of group i to the enthalpy of melting (kJ/mol), m_j is the contribution of proximity factor j to the enthalpy of melting (kJ/mol).

The molecular fragmentation scheme for calculating the enthalpy of melting is based on the concept of an isolating carbon. An isolating carbon is a carbon that is not doubly or triply bonded to a hetero atom. Each molecular fragment is defined as the smallest group of atoms (consisting of all carbons, hydrogen, and hetero atoms,

including their non-bonded electrons) that are not separated by an isolating carbon. The use of this concept allows identifying a wide array of molecular fragments ranging from single atoms to large polyatomic groups (Jain and Yalkowsky 2006). Interested readers should familiarize with the excellent contributions of Jain et al. (2004) and Jain and Yalkowsky (2006) devoted to the estimation of melting point, enthalpy and entropy of melting of organic compounds.

Provided the melting temperature is known, then it is possible to estimate ΔH^{fus} from the following empirical equation:

$$\Delta H^{\text{fus}} = 4.184kT^{fus} \tag{4.36}$$

where k is an empirical parameter related to the properties of the solute, and for organic compounds it is usually in the range 10–16 (Chen and Ma 2004).

Yet another possibility is to estimate the sublimation enthalpy solely on the basis of the value for ΔH^{vap}, taking into consideration that ΔH^{fus} is usually less than one quarter of the sum given by Eq. (4.33) (Prausnitz et al. 1999).

Solid molar volume. If no experimental data for the solid molar volume of a compound is available, it can be estimated applying for example the method of Bondi (1964).

Another possibility is to use the method of Fedors (1974). The author presented a very simple group contribution approach to estimate molar volume of pure substances, by adding atomic and group contributions (Δv_i):

$$V = \sum_i \Delta v_i \tag{4.37}$$

Of special interest is the large number of atoms and groups for which the additive increments have been evaluated. The contributions reported are applicable at a temperature of 25 °C. Furthermore, it has been found that the molar volume for cyclic compounds can be estimated from the properties of a linear compound having the same chemical structure, and by adding a cyclization increment. Experience using this method has shown that deviations between the experimentally measured V and the estimated values are generally less than 10 %. For example, application of Fedors method to the prediction of β-carotene molar volume at 25 °C resulted in deviation of just 7.5 %.

Goodman et al. (2004), in an effort to upgrade the methods used by the DIPPR database project for estimation of solid properties, reviewed those for estimating solid density. It was found that a simple ratio of the liquid density at the triple point (deemed to be readily available), and density of the equilibrium solid phase at the triple point:

$$\frac{\rho_{\text{S}}(T_{\text{t}})}{\rho_{\text{L}}(T_{\text{t}})} = 1.17 \tag{4.38}$$

is most reliable.

Goodman et al. (2004) extended Eq. (4.38) to include temperature dependence for solid density from T_t to substantially lower temperatures. The extended relationship is:

$$\rho^{\text{solid}}(T) = \left(1.28 - 0.16\frac{T}{T_t}\right)\rho^{\text{liquid}}(T_t) \tag{4.39}$$

where ρ^{solid} is the solid density, and $\rho^{\text{liquid}}(T_t)$ is the liquid density at the triple point, which is calculated from an EoS.

4.5.3 *Uncertainties in Thermophysical Properties of Pure Compounds*

Needless to say that the lack of reliable experimental data for most of the solutes extracted from vegetable matrices forces the application of correlations and estimation methods. The latter, of course, inevitably leads to uncertainties in the values obtained.

Within any model class and any mixture category, the mixture condition (temperature, pressure and/or composition) determines the sensitivity and/or applicability of the model equation. It is therefore obvious that different categories of mixtures will have different sets of model parameters.

EoS as Thermodynamic Model. The thermodynamic model parameters for an EoS are usually the component critical temperature, pressure, acentric factor, plus the coefficients for the ideal-gas heat capacity. The mixture parameters in an EoS are determined from suitable mixing rules which are functions of pure component properties, conditions of operation and binary parameters (determined using mixture properties).

It is well known that small errors in critical properties used in equations of state affect the quality of final results sometimes, in a dramatic fashion. For example, the effect of errors in the critical temperature of different compounds using the Peng–Robinson equation of state shows that if the critical temperature is under-estimated by 2 % from its accepted value, errors in vapour pressure between (20 and 60) % for several evaluated compounds are obtained.

The uncertainties in the values of the parameters of the pure components can seriously influence the quality of phase equilibria predictions at high pressures. Pfohl et al. (1998) demonstrated that the over-prediction of critical pressure and temperature of pure components can lead to erroneous phase splitting in the region close to the supercritical solvent's critical point in mixtures. Vafai et al. (1993) pointed out the importance of accurate values of the solid molar volume for modelling the phase equilibria, and demonstrated that at 40 MPa and 310 K, an 11 % error in the solid molar volume of naphthalene would correspond to a 21 % error in the enhancement factor. Somewhat contradicting are the observations of

Coutsikos et al. (2003) who performed predictions with the LCVM model for two systems containing highly complex compounds, namely cholesterol and naproxen. They demonstrated that the model failed in these cases, resulting in a very large overestimation of the solubilities by often two or more orders-of-magnitude. The authors conclude that the poor predictions are not very much affected by the critical properties used, which are not known accurately. To substantiate their findings they give as example cholesterol for which the performance of the model does not change significantly regardless of the values for the critical parameters and acentric factor applied. The authors pointed that unlike the effect of critical properties, the predicted solubilities are affected significantly by the value used for the vapour pressure of the solid. This is usually the case when modelling food-type components, which are generally large molecules with very low vapour pressures, and thus some investigators consider vapour pressures as fitting parameters. Still, as mentioned previously, such approach *does not guarantee* low deviations between correlated results and experimental data.

Gani and Constantinou (1996) considered the supercritical extraction of β-carotene with supercritical CO_2. Since the experimental values of critical properties for β-carotene are not available, they applied the methods of Lydersen (1955), Joback and Reid (1987) and Constantinou and Gani (1994) to predict the missing properties. The following values were obtained: $T_c = 674.4$ K, $P_c = 8.36$ atm and $\omega = 4.8$ with Lydersen method; $T_c = 1{,}480.9$ K, $P_c = 6.07$ atm with Joback method and, $T_c = 905.3$ K, $P_c = 6.95$ atm and $\omega = 1.46$ with Constantinou and Gani method. The authors also demonstrated that if, for example, solubility data was employed to "fit" the above properties (an approach that is *not* recommended) the following values were obtained: $T_c = 647.9$ K, $P_c = 15.2$ atm and $\omega = 0.411$.

The influence of the method selected to estimate the thermophysical properties required can be seen from the simulation/design of the process. The calculated costs of operation differ by more than 50 % for the "fitted" thermophysical properties of β-carotene and those obtained with Constantinou and Gani method (Gani and Constantinou 1996).

Sovova et al. (2001) show on the example of the triolein + CO_2 binary that the uncertainties in the values of the critical parameters might have a dramatic effect on the phase behaviour calculations and on the predicted extent of the vapour-liquid region. Thus, applying some of the estimations of triolein critical parameters, the system was erroneously predicted to be homogeneous at very low values of the pressure.

However, it should be underlined that to perform a detailed analysis of the influence of the thermophysical properties uncertainties on the phase behaviour predictions and calculations of systems of interest to the food industry (or any other complex systems to that matter) is a demanding task, which faces many challenges and must be explored from many different angles.

One of the challenges is a result of the fact that there are very big deviations between the pure component parameters values estimated and reported by the different authors applying different methods. In view of this a range of effective methods to estimate the solutes properties should be available within the TMF.

Then, as pointed out by Hajipour and Satyro (2011), Monte Carlo techniques should be used to evaluate the error propagation from uncertainties in thermophysical properties values and their effect on process simulation and design results.

Group-Contributions Models. Group Contribution models like UNIFAC, UNIQUAC, NRTL do not require critical parameters of the pure components. The model parameters for those models are the molecule (or group) surface area and volume and, binary molecular (or group) interaction parameters. Still, the influence of those parameters on the quality of phase equilibria predictions and calculations can be considerable.

In the light of the above, some of the practical problems associated with the application of the UNIFAC based mixing rules can be briefly summarised as follows:

Many solid solute compound molecules extracted from natural matrices are more complex than the molecules normally encountered in gas-liquid equilibria. In addition, these molecules sometimes contain functional groups for which UNIFAC parameters are unavailable. And even if all group definitions exist, a subset of the relevant parameters is unavailable.

Furthermore, group-contribution methods treat the light gases, like CO_2, as individual groups, and hence the volume and surface area parameters for these groups must be determined. In some cases, as pointed out by Fornari et al. (2010), these structural parameters are estimated using semi-theoretical methods like those of Bondi (1964), and Apostolou et al. (1995) but their values are essentially arbitrary and differ from those given by Dahl et al. (1991), the size parameters of the latter being about twice the size of the gases of Apostolou et al. (1995). There has not been a thorough analysis of which values are the best. In addition, these values must be set before the optimum values of the group interaction parameters of the UNIFAC model can be found, and hence any deficiency that exists in the selection of these structural parameters will inevitably affect the group interaction parameters and the results of the phase equilibria prediction and modelling (Orbey and Sandler 1998).

Finally, the interaction parameters between groups are determined by correlation of the model to experimental data. For UNIFAC parameters, for example, more than 60 different functional groups are determined simultaneously in an extensive optimization where the total deviation between the UNIFAC model and huge amounts of experimental data is minimized. Because of the complexity of this optimization, parameter values are primarily correlation values rather than describing the actual physics in each particular interaction, and of course this limits the possibility to extrapolate the use into new situations. Furthermore, the values of the UNIFAC parameters to be used have been determined from vapour-liquid equilibrium data, because these are the most extensive data that are available. From a physical point of view these interaction parameters should be equally useful for solid–liquid equilibria. However, because of deviations from the underlying assumptions and because of the complexity of the parameter determination, the extrapolation to solid–liquid systems is not necessarily valid.

Table 4.1 Different sets of squalene pure component properties

Property	Set				
	I	II	III	IV	V
T_c (K)	886.1[a]	838.1[b]	862.5[c]	862.5[c]	862.5[c]
P_c (bar)	9.36[a]	6.50[b]	9.49[c]	9.36[a]	6.50[b]
d_c ()	9.336[b]	9.244[b]	9.336[b]	9.336[b]	9.336[b]
d_c from Eq. (4.27)	8.896	9.861	8.775	8.816	9.955

[a] Wakeham et al. (2002)
[b] Fornari (2007)
[c] Somayajulu (1989)

Representatives of thermodynamic models which incorporate into the EoSs activity coefficient models (e.g. MHV2, PSRK, GC-EoS) require pure components thermophysical parameters and group-group data. There are some limited data in the literature demonstrating the influence of properties uncertainties on the phase behaviour predictions and solubility correlations applying those models, see for example Sovova et al. (2001), Martinez-Correa et al. (2010), etc.

In their contribution, Martinez-Correa et al. (2010) used the Group Contribution Equation of State (GC-EoS) to calculate the solubility of squalene in supercritical CO_2. The authors studied the effect of different sets of squalene pure component parameters (critical temperature, critical pressure and critical hard sphere diameter) on the solubility calculations. The different sets employed, together with the estimation methods applied, are given in Table 4.1.

On the one hand, when the same value for the critical hard sphere diameter (d_c) but different values for T_c and P_c (property sets I, III, IV and V) were used there were no marked differences with respect to the solubilities predicted by the GC-EoS model. Thus, at a given temperature, very similar average absolute relative deviations were obtained when $d_c = 9.336$, despite the different T_c and P_c values employed. On the other hand, when the same T_c and P_c values were used (critical temperature and pressure given in Set II) the thermodynamic model was very sensitive to the value of the squalene d_c parameter; as a result, the deviations obtained with $d_c = 9.336$ were almost half of those obtained with a just 5 % lower d_c value.

Conclusions

Green high pressure fluid technology for food processing is a part of the sustainable development and industrial strategy in the twenty-first century. In recent years there is a considerable expansion of foods for specific health use, food supplements and nutraceuticals' market. Many of the target bioactive ingredients originate from natural vegetable sources. The efforts of researchers and engineers should be focused on meeting the challenges of

(continued)

(continued)

developing novel, efficient and sustainable production procedures, such as those applying high pressure processing.

The design and development of such efficient green processes are, however, strongly dependent on the availability of reliable physical and thermodynamic properties data of the compounds involved in the process. Nevertheless, often it is not possible to find experimental values of some/all of the properties required, because it is either time consuming (and often expensive) to measure them or because they may not be experimentally accessible. Hence, availability of efficient methods to estimate thermophysical properties of pure substances becomes critically important.

The state-of-the art in this field includes different methods based on: numerical descriptors of chemical structure; on establishing relationships/correlations between structure and properties of molecules (quantitative structure–property relationships); the use of the group contribution approach.

In general, group-contribution methods are the oldest but still very much the favourite tool. The simple first-order group-contribution approach has been traditionally employed to estimate pure component properties (boiling point, melting point, critical properties, acentric factor, etc.) of hydrocarbons and simple derivative families (ethers, esters, aldehydes, etc.). The methods belonging to that group provide quick estimates, require relatively small data set, but are of questionable accuracy—e.g. cannot distinguish among isomers. Furthermore, most of the existing first-order methods do not include suitable groups for representing complex molecules such as the ones of biochemical or environmental importance.

To overcome these limitations, two- and three-level group estimation methods, as well as other more elaborate methods, are recommended. For example, the second level involves groups that permit a better description of proximity effects and differentiation among isomers, and can deal with polar or nonpolar compounds of medium size and aromatic or cycloaliphatic compounds with only one ring and several substituents. The third level has groups that provide more structural information, allowing estimation of complex heterocyclic and large molecular weight polyfunctional acyclic compounds.

Finally, it should be underlined that each method's sensitivity and/or applicability depends on the mixture condition (temperature, pressure and/or composition) which inevitably leads to uncertainties in the properties' values obtained. The latter have a profound influence on the reliability and robustness of the mixture phase behaviour correlation/predictions, which, in turn affect the simulation, design and optimization of high pressure food processes. Thus, special considerations have to be taken to the impact of these uncertainties as was outlined in this chapter.

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Part II
Advances in High Pressure Food Processing

Chapter 5
Particle Formation of Food Ingredients by Supercritical Fluid Technology

Irene Rodríguez-Meizoso and Merichel Plaza

5.1 Introduction

A food ingredient is "any substance, including a food additive, employed in the manufacture or preparation of a food and present in the final product, even in its modified form" (General Standard for the Labelling of Prepackaged Foods, Section 4—Labelling of Prepackaged Foods (CODEC SRAN 1-1985)). There is a group of food ingredients that is called *functional ingredients*. These ingredients are responsible for improving the state health or well-being, or reducing the risk of disease (Diplock et al. 1999). This chapter focuses on food ingredients with bioactive effect, considering different families such as phenolic compounds, lipids and carotenoids, vitamins, amino acids, peptides and proteins (enzymes), probiotics (bacteria), carbohydrates and minerals.

Many of these compounds are sensitive to temperature, light and oxygen. These factors may trigger reactions leading to molecular structural changes (denaturalization of protein, reactions of unsaturated chemical bonds, oxidation, etc.), which may result in undesirable quality of the food or reduced/lost bioactivity. Furthermore, many of these bioactive ingredients are insoluble or marginally soluble in aqueous solutions. Development of delivery systems may improve the stability of bioactive ingredients. For instance, micro- and nano-encapsulation have been used for the delivery of active substances that are susceptible to degradation. The coating material of encapsulates can act as a protective layer against aggressive agents, improving their stability. Besides, decreasing the particle size facilitates homogenization of mixtures and dosage. Also, smaller particle size involves higher surface area, which is associated with higher solubility in water and improved bioavailability (Chaudhary et al. 2012).

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Particle formation into micro- and nanoparticles generates increasing interests among food scientists. The greatest requirement in particle formation is the particle size and morphology, which determine the potential application of the bioactive compounds. Micro- and nanoparticles are defined as particulate dispersions or solid particles with size in the range of (1–100) μm and (10–1,000) nm, respectively, although these ranges may vary between different authors and disciplines. Micro- and nanoparticles can be obtained by different techniques. Conventional techniques (jet and ball milling, spray-drying, solute recrystallization, coacervation, freeze-drying, interfacial polymerization) present several drawbacks such as excessive use of solvents, thermal and chemical solute degradation, structural changes, high residual solvent concentration, high shear forces, electrostatic phenomena and mainly, difficulty of controlling the particle size and particle size distribution during processing, so these techniques for particle formation do not offer much control on the final product (He et al. 2004; Nunes and Duarte 2011).

The processes to produce micro- or nanoparticles can be classified into the "top-down" and "bottom-up" approaches. "Top-down" approaches involve the size-reduction of large particles to micro- or nanometer range. In contrast to "top-down" methods, the "bottom-up" approaches generate micro- or nanoparticles by building them from molecules in solution. The majority of supercritical fluid (SCF) processes to produce micro- or nanoparticles are carried out via a "bottom-up" approach, except perhaps for certain processes based on the PGSS™ (particles from gas-saturated solution, see Sect. 5.4) technique, when the compound of interest is present as a solid that melts upon its contact with the SCF. Should the term micronization refer only to a decrease in size, it must be used carefully in the case of SCF technologies and perhaps replaced by terms like particle formation, particle precipitation, particle formulation or recrystallization.

SCF techniques for particle formation have been developed with focus on pharmaceutical compounds. Their application in food science has increased over the last 10 years, which is reflected in more than twice as many publications as in the previous decade. The strength of SCFs in particle formation applications comes from their unique properties related to solvent power and fast diffusion rates. The solvent power of SCFs is related to density, and it can be tuned within several orders of magnitude by changes in pressure and temperature, as well as by the addition of entrainers (cosolvents, modifiers). This fact can be exploited to create highly supersaturated systems that will quickly evolve towards precipitation of small particles/crystals. At the same time, due to the high diffusion coefficients of SCFs, the supersaturation conditions are created fast in the whole system, enabling homogeneous nucleation and growth, and thus producing low particle size distribution. Whether a compound of interest will precipitate or crystallize in certain morphology is related to the degree of supersaturation. It is inferred that a combination of process parameters and physicochemical properties of the compound of interest play a crucial role in the morphology of the particles obtained. So far, the relation between process parameters, size, morphology and composition of the particles is still unpredictable and case-specific, and the desired product is achieved by trial-and-error. A wide range of morphologies can be achieved for the different

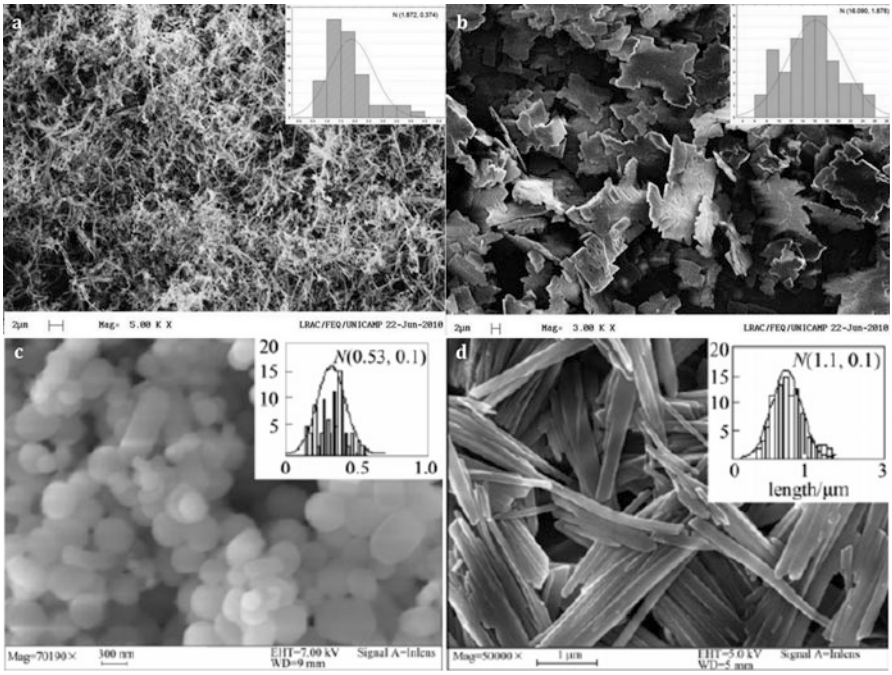

Fig. 5.1 Scanning electron micrograph of quercetin needle-like particles obtained by SAS (**a**), β-carotene leaf-like particles obtained by SAS (**b**), astaxanthin spherical-like particles obtained by RESS (**c**), and quercetin flake-like particles obtained by SEDS (**d**). Reproduced with permission of Santos and Meireles (2013) and Can et al. (2009)

food ingredients using SCF technology, from needles to spheres and leaf-like shapes (see Fig. 5.1).

Fluids like ammonia, propane, diethyl ether and nitrous oxide have been used as the SCF for particle formation purposes in general. However, carbon dioxide is by far the most common one due to several reasons. It is one of the safest SCFs because it is non-flammable and non-toxic. CO_2 is chemically inert and it reaches the supercritical state at rather low critical temperature (31 °C) and moderate critical pressure (7.3 MPa) compared to other supercritical fluids, which makes it particularly suitable for the processing of thermolabile and/or oxidative compounds such as antioxidants and aromas relevant for the food industry. Furthermore, it is considered a GRAS (generally recognized as safe) solvent by the FDA (American Food and Drug Administration) and accepted by the EFSA (European Food Safety Authority).

Particle formation techniques based on SCFs are mentioned in several books with focus on drug manufacture and nanotechnology, but the best compilations and descriptions of techniques are found in review articles, patents and original research articles like those by Jung and Perrut (2001), Cocero et al. (2009) and Reverchon and Adami (2006). The review by Weidner (2009), focused on food applications, is

of particular relevance for this chapter. The reader will find many acronyms referring to particle formation process with supercritical CO_2 ($SCCO_2$), even though they might be based on the same technique. We can conveniently classify the techniques in three blocks, according to the role of the $SCCO_2$:

- $SCCO_2$ as a solvent, represented by the RESS (rapid expansion of supercritical solutions) process
- $SCCO_2$ as an anti-solvent, represented by the SAS (supercritical antisolvent) process
- $SCCO_2$ as a solute/dispersant medium, represented by the PGSS™ (particles from gas saturated solutions) process

The different techniques and processes, as well as their application in food technology, will be described in the following sections.

To be able to choose a particle formation technique suitable for a specific application it is necessary to understand the phase behavior of solids and liquids in SCFs, and solid–liquid-SCF mixtures. Prediction of phase diagrams of $SCCO_2$ systems is difficult due to the non-classical behavior of this region and the lack of critical parameters for most solutes. Modeling such systems is therefore limited and process development is commonly performed by trial-and-error.

A good starting point for process development is to discern if the compound of interest is soluble or not in the SCF. For the particular case of $SCCO_2$, solubility is not a trivial matter. To estimate if a compound might be soluble in $SCCO_2$ it is convenient to think of $SCCO_2$ as a solvent with nonpolar nature that will be able to dissolve nonpolar compounds. However, $SCCO_2$ possesses a quadrupole moment, which enables the solvation of molecules that can interact with the CO_2 molecule through electron donor/acceptor interactions; that is, certain aldehydes, ketones, alcohols and esters. But the amount of compound that can be dissolved depends on the solvent strength of $SCCO_2$. Solvent strength is related to density, meaning that higher pressures usually lead to a higher solubility of the solute while higher temperatures have the opposite effect. However, increasing the temperature can increase solubility due to increased vapor pressure of the compound of interest, as long as the pressure is high enough. There are substantial amounts of solubility data available for consultation in books (Gupta and Shim 2007) and in scientific journals like Fluid Phase Equilibria, Journal of Chemical Engineering Data, Journal of Supercritical Fluids and Journal of Chemical Thermodynamics. Advanced theories to predict solubility such as the Hansen Solubility Parameters are limited in $SCCO_2$ systems since there is no rigorous way to calculate the dependence of the dispersion, dipole and especially chemical interactions (meaning hydrogen bonding and Lewis acid–base complexation) with pressure and temperature (Hansen 2000). Modeling is currently approached using either equations of state (EoS) like Peng-Robinson EoS and Soave-Redlich-Kwong EoS combined with mixing rules, or semi-empirical equations, but their predictive power is poor. For a recent review on the modeling of solubility in SCFs see Skerget et al. (2011). Experimental determination of phase equilibrium is still essential either as a source of data for process

development or for the improvement of predictive models. Several methods to determine solubility of compounds in high-pressure systems have been described and systematically summarized in literature (Dohrn et al. 2012).

However, if the compound is not soluble in $SCCO_2$, there are several options. In the case of liquid compounds and melts, $SCCO_2$ can be used as a solute that is dissolved into the liquid phase and takes the role of a physical dispersant in an atomization step, i.e. in a PGSS-like process. In this case, it is advantageous to know the solubility of $SCCO_2$ in the liquid. In the case of solid compounds, it is necessary to find a liquid solvent able to dissolve the compound of interest and at the same time be miscible with $SCCO_2$. The $SCCO_2$ will act as an antisolvent upon mixing, forcing precipitation of the solute. The choice of solvent must also consider the phase diagram of the ternary mixture solute-solvent-CO_2, as the same solute might behave very different depending on the solvent. The mixture solute-solvent-CO_2 should be such that the phase diagram shows a 2-phase solid–fluid region within operational conditions, where the solute is forced to precipitate in high yield. The phase behavior of ternary mixtures must be studied experimentally, although several efforts have been made to develop predictive tools (Su 2013; Kikic et al. 2010; Colussi et al. 2006; Shariati and Peters 2002).

If the solute is insoluble in the mixture and does not exhibit strong interactions with the solvent, we can assume that the presence of the solute does not affect the solvent-CO_2 equilibrium, and it is therefore possible to consider only the binary system solvent-CO_2, rather than the ternary phase diagram. For such simplified systems, the 2-phase region for precipitation can be found above the critical point of the mixture solvent-CO_2, where $SCCO_2$ and solvent form a single phase. To estimate the critical point of the mixture the reader can use specialized software like GPEC (Cismondi et al. 2009) or find experimental data in the literature.

For further information about phase diagrams of solute-SCF mixtures, including solubility of solids and liquids in SCFs, the authors refer to McHugh and Krukonis (1994), and Chap. 1 of this book.

In the case of water, the mixture critical point is reached at such high temperatures and pressures that the compounds of interest would degrade before precipitation. For this reason, in the case of aqueous solutions, $SCCO_2$ will not play an antisolvent role but behave as a physical dispersant to enhance an atomization step followed by drying with the help of an adjutant.

An additional case can be found with samples in the form of a suspension. These cases might be common in the preparation of composites and encapsulates, and they can be processed by any of the three basic techniques described above, providing appropriate use of solvents and slight modifications in the equipment to account for clogging problems.

As a guide for readers and future users, Table 5.1 provides a summary of the techniques used for particle formation in food technology, organized for family of compounds.

In a lab-scale, the equipment used to perform particle formation of food ingredients includes mainly home-built apparatus, with the exceptions of a modified

Table 5.1 Compilation of the works found in literature about the particle formation of food ingredients by supercritical fluid technology, organized by family of compounds

Compound	Technique	Particle size	Morphology	References
Carotenoids				
Astaxanthin	RESS	0.5 μm	Spherical	Can et al. (2009)
	SEDS-PA	0.5–6.9 μm	–	Hong et al. (2009)
β-carotene	SAS	3.288 μm	Leaf	Santos and Meireles (2013)
	SAS (microencapsulation with polyethylene glycol)	100–200 μm	Prismatic	Martín et al. (2007)
	PGSS (microencapsulation with lecithin)	10–500 μm	Spherical	De Paz et al. (2012)
Lutein	SAS (nanoencapsulation with hydroxypropylmethyl cellulose phthalate)	163–219 nm	–	Koushan et al. (2013)
	SAS (microencapsulation with polyethylene glycol)	50 μm	Spherical and amorphous	Martín et al. (2007)
Bixin	SAS (microencapsulation with polyethylene glycol)	33 μm	Flake, bar, etc.	Santos and Meireles (2013)
Vitamins				
Ascorbyldipalmitate	RESOLV	80 nm	–	Sonkaew et al. (2012)
Retinylpalmitate	RESOLV (nanoencapsulation with poly(L-lactide))	40–110 nm	Spherical	Sane and Limtrakul (2009)
Phenolic compounds				
Benzoic acid	RESS	0.8–1.2 μm	–	Türk (1999)
Curcumin	RESOLV	50 nm	–	Sonkaew et al. (2012)
Rutin	RESS (microencapsulation with polyethylene glycol)	42.94 μm	Amorphous	Santos and Meireles (2013)
Anthocyanins	RESS (microencapsulation with polyethylene glycol)	40–110 nm	Spherical	Santos and Meireles (2013)
3,5-diprenyl-4-hydroxycinnamic acid (from propolis extract)	SAS	2 μm	–	Wu et al. (2009)

(continued)

Table 5.1 (continued)

Compound	Technique	Particle size	Morphology	References
Quercetin	SAS	1.872 μm	Needle	Santos and Meireles (2013)
	SEDS	1–3 μm	Needle or flakes	Can et al. (2009)
Phenolic diterpenes (rosemary)	WEPO	< 93 μm	Agglomerates	Rodríguez-Meizoso et al. (2012), Herrero et al. (2010)
Quercetin derivatives (onion)	WEPO	250 nm to 4 μm	Spherical	Andersson et al. (2012)
Probiotics				
Bifidobacteriumlongum	PGSS (encapsulation with interpolymer complex)	168 μm	Monolithic foam structure	Moolman et al. (2006)
	PGSS (encapsulation with interpolymer complex with viscosity modifier)	6.9 μm	Monolithic foam structure	Moolman et al. (2006)
Bifidobacteriumlactis Bb12	PGSS (encapsulation with interpolymer complex)	166.1 μm	Foam structure	Mamvura et al. (2011)
Proteins				
Corn zein	SAS	79–105.5 nm	–	Zhong et al. (2008)
Sterols				
Cholesterol	RESS	< 0.35 μm	–	Türk (1999)
β-Sitosterol	RESS	100–200 nm	Quasispherical and needles	Türk et al. (2002)
	RESSAS	5–200 nm	–	Türk et al. (2002)
Phytosterol	RESSAS	< 500 nm	–	Türk and Lietzow (2004)
Lipids				
Soy lecithin (phospholipids)	SAS	1–40 μm	Amorphous spherical and aggregated particles	Magnan et al. (2000)
Anhydrous milk fat	ScMM	10–40 μm	Spherical hollow and sponge	Lubary et al. (2011)
Diacylglycerol-based modified milk fat	ScMM	10–40 μm	Spherical hollow and sponge	Lubary et al. (2011)

(continued)

Table 5.1 (continued)

Compound	Technique	Particle size	Morphology	References
Carbohydrates (prebiotics)				
Lactulose	SSI (impregnation on chitosan)	1–2 mm	Spherical and scaffolds	Diez-Municio et al. (2011)

supercritical fluid extraction system to perform RESS from Waters (Milford, MA, USA). In an industrial scale, particle formation of food ingredients is performed by companies like Natex and Feye Con.

5.2 $SCCO_2$ as Solvent

5.2.1 RESS (Rapid Expansion of Supercritical Solutions) Technique and Related Processes

RESS is the preferred technique when the compound of interest is soluble in $SCCO_2$. The solute is put in contact with $SCCO_2$ in a pressurized vessel and dissolved at an operational pressure and temperature. Then, the solution is depressurized through a nozzle into a lower-pressure chamber, causing the expansion of the SCF and precipitation of the solute.

The driving force for precipitation is the solubility difference between the solute in the SCF (pre-expansion conditions) and in the low-pressure fluid (during expansion). The supersaturation ratio (**y**/**y***) is defined as the ratio of the mole fraction of the solute in the solvent at prevailing pressure and temperature during the expansion (**y**) to the mole fraction of the solute in the solvent at pre-expansion conditions (**y***). As the pressure drops, CO_2 becomes gas, loses its solvent power and the solute precipitates to compensate for the large supersaturation ratio created during the expansion of the supercritical mixture.

The high supersaturation ratio is responsible for the small particle size of the precipitate, as many nuclei are created. Supersaturation occurs fast and homogenously throughout the sample, which is responsible for the small particle size distribution of the precipitate. Expansion is also responsible for a fast drop in temperature. The drop of temperature and pressure, together with the melting temperature of the solute, dictate if the solid particles are formed directly from the SCF (crossing a fluid-solid phase boundary in the pressure-temperature phase diagram) or if the system first separates into two liquid phases (crossing a liquid-liquid phase boundary in the pressure-temperature phase diagram) followed by solidification. The pathway followed from a one-phase (supercritical) system to the phase separation influences the size and morphology of the particles, and it is of

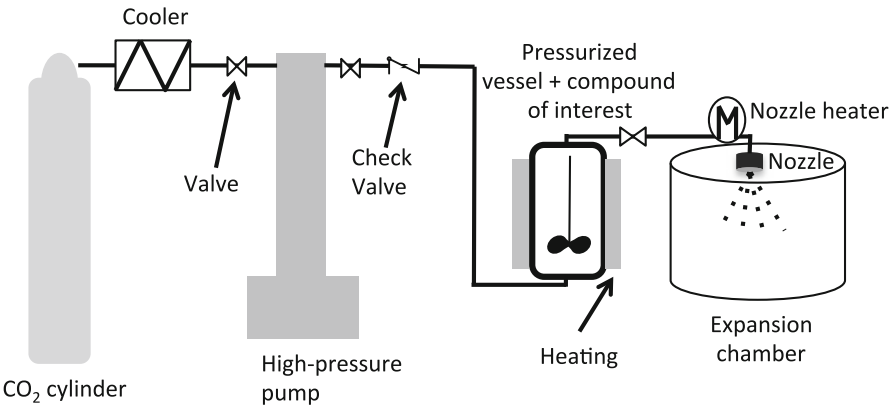

Fig. 5.2 Schematic diagram of a RESS setup. The expansion vessel can be empty (RESS), contain organic (RESOLV) or aqueous solutions (RESSAS)

great importance in the case of semi-crystalline polymers and other crystalline compounds, as it might affect the degree of crystallinity and polymorph structure of the final particles.

Temperature and pressure in the pre-expansion and expansion chamber, nozzle geometry, solubility and melting point of the solute are important parameters to optimize. The extent to which these parameters affect the size, size distribution and morphology of the produced particles is not trivial and still case specific. For a lead reference on the fundamentals of the technique see Türk (2009).

Figure 5.2 shows a schematic diagram of a RESS setup. It is basically composed of solvent cylinder, cooler, high-pressure pump, high-pressure vessel (usually with stirring), vessel heater, nozzle, nozzle heater and expansion chamber.

RESS is usually performed in batch mode, in the sense that the solute is introduced in the pre-expansion vessel and given time to dissolve in $SCCO_2$ before expansion. A continuous mode, where solute and solvent are constantly introduced in the pre-expansion vessel and sprayed into a collection chamber, is mainly limited by the solvation kinetics of the compound of interest in $SCCO_2$.

The original RESS process does not involve the use of organic solvents, which is considered an advantage from an environmental point of view, as well as to ensure no solvent residues in the final product. But the process is limited to compounds with high solubility in $SCCO_2$. To overcome this problem, Sun et al. (2002) developed the n-RESS process. In the n-RESS process, the compound of interest is dissolved in a mixture of $SCCO_2$-cosolvent, providing that the solute is not soluble in the cosolvent at ambient conditions.

RESOLV (rapid expansion of supercritical solution into liquid solvents) is another variant of the RESS process, developed to obtain nano-sized particles (Sun et al. 2002). RESOLV consists of expanding the supercritical solution into a liquid solvent or solution instead of into ambient air. The solvent may contain a stabilizing agent like a surfactant. The contact with the solvent avoids coagulation

and growth of the particles. The process is sometimes called RESSAS (rapid expansion from supercritical to aqueous solutions) when the receiving solvent is water or an aqueous solution (Young et al. 2000). However, there is little consensus among the different names and the reader will often find the expansion into aqueous solutions referred to as RESOLV process.

The CORESS process is a variant of RESS used to produce composite particles. In the CORESS, both compound of interest and encapsulating agent are dissolved in $SCCO_2$ and co-sprayed, leading to simultaneous co-precipitation of the solutes and formation of composite powders (Türk et al. 2006).

Applications. The formation of particles through RESS has been used for the recovery of high value functional ingredients. For instance, Türk (1999) produced small organic particles by RESS, like benzoic acid and cholesterol. Benzoic acid is a phenolic acid with antioxidant properties, while cholesterol is an essential sterol for all animal life. RESS experiments were carried out at temperatures up to 600 K and pressures up to 60 MPa. These experiments led to particle sizes in the range of (0.8 and 1.2) μm for benzoic acid and always lower than 0.35 μm in the case of cholesterol. Later, Türk et al. (2002) used RESS to improve the bioavailability of β-sitosterol. With this purpose, nanoparticles of β-sitosterol were formed by RESS process into air and into aqueous solution (so-called RESSAS). β-sitosterol is a plant sterol (phytosterol) highly appreciated for its positive health effects, as its intake decreases the levels of total cholesterol and low-density lipoprotein cholesterol (LDL) in blood (Ras et al. 2013). Stable suspensions of nanoscale particles of β-sitosterol were produced by RESS into aqueous solutions. The particles sizes of β-sitosterol in the aqueous solution (5–200 nm) were smaller or equal to those produced by RESS into air (100–200 nm). The β-sitosterol particles obtained by RESS into air presented spongy structure with high surface area, suitable to improve the bioavailability of water-insoluble bioactive compounds.

Using the RESSAS technique, Türk and Lietzow (2004) synthesized phytosterol nanoparticles (below 500 nm) with long-term stability. The phytosterols, as explained above, are present in foods and reduce the intestinal cholesterol absorption, thus lowering plasma LDL cholesterol in humans (Plösch et al. 2006), making it a very interesting functional ingredient. In this study, a supercritical phytosterol/CO_2 mixture was expanded into an aqueous surfactant solution. The experiment conditions were a pre-expansion temperature 388 K, pre-expansion pressure 20 MPa, nozzle temperature 398 K and solution temperature 303 K. Furthermore, four different surfactants (Solutol HS15, Lutrol F68, Tween 80 and sodium lauryl sulfate (SLS)) were tested to avoid growth and agglomeration of the submicron particles resulting from collisions in the free jet. They observed that the surfactant types and concentration influenced the particle size. For instance, the smallest particle sizes (35–55 nm) were obtained using SLS surfactant at the highest concentration studied (1.1 wt%). The results in this study demonstrated that RESSAS is a promising process for stabilizing submicron particles of bioactive compounds in aqueous solutions.

RESS process has been used for carotenoid particle formation. Carotenoids are a family of natural pigments that are of great interest for the food industry as

colorants and due to their high antioxidant capacity (Higuera-Ciapara et al. 2006). Can et al. (2009) carried out the formation of astaxanthin particles by RESS. The mean particle size of astaxanthin decreased as the pressure increased from (15–30) MPa. The reason may be that increasing pre-expansion pressure decreases the critical nucleus size (the specific size determined by the competition between the aggregate curvature and the free energy favoring the growth of the new phase) and thus produces smaller particles. Also, the effect of pre-expansion temperature was investigated at (313.15 and 333.15) K, at pre-expansion pressures of (20 and 30) MPa. They observed that smaller particles were produced at higher pre-expansion temperature. They compared the RESS technique with conventional crystallization with n-hexane. The morphology of the particles obtained under different experimental conditions was sphere-like, opposed to unprocessed amorphous astaxanthin crystals. The particle size from the RESS (0.3–0.8 μm) was typically 10 times smaller than in conventional crystallization with a mean size of 5 μm.

RESS into aqueous solutions, referred to as RESOLV by Sonkaew et al. (2012), was used for the production of curcumin and ascorbyldipalmitate (ADP) nanoparticles with average sizes of approx. (50 and 80) nm, respectively (Sonkaew et al. 2012). These compounds have important biological activities. For instance, curcumin is a phenolic compound derived from the root of turmeric. Curcumin has powerful antioxidant properties (Srinivasan 2014). ADP is a fatty ester derivative of ascorbic acid. ADP has been extensively used as an additive to prevent oxidation in foods, pharmaceuticals and cosmetics (Moribe et al. 2010). In these experiments, the pre-expansion vessel was charged with 0.097 g of curcumin or ADP, 16.1 g of ethanol and 16.1 g of CO_2. The mixture was pressurized to 17.3 MPa at (318.15–323.15) K. The solution was expanded through the nozzle into 50 mL of 0.1 wt% pluronic F127 aqueous solution as stabilizer. The curcumin and ADP nanoparticles showed higher antioxidant activities than those of unprocessed curcumin and ADP. Moreover, the authors incorporated the nanoparticles into cellulose-based films by using 3 wt% methyl cellulose as receiving solution, as a promising technology to create antioxidant packaging films.

Other possible application of RESS is the formation of polymeric microcapsules of bioactive compounds. The encapsulation of bioactive compounds presents several advantages, such as controlled delivery of the bioactive into its targeted media. Also, the coating material can act as a protective layer against aggressive agents and the compounds encapsulated can be easier to handle and dose than pure compounds (Cocero et al. 2009). For instance, Santos and Meireles (2013) used RESS to obtain microcapsules of rutin and anthocyanin-rich extract (core material) with polyethylene glycol (PEG) with a mean molecular weight of 10,000 g/mol as encapsulating material and ethanol as cosolvent. The operational conditions employed in this work were 20 MPa of pre-expansion pressure, 313.15 K of temperature, and concentrations of 27.1 % (w/w) of ethanol and 8.1 % (w/w) of PEG. Two mass ratios between core material and PEG of 1:2 and 1:10 were investigated. They observed that a decrease in the concentration of core material resulted in a decrease in the encapsulation efficiency and in the particle size. Rutin particles (mass ratio of 1:10) were well distributed in amorphous microcapsules and the mean particle size

was 42.94 μm. The amount of residual ethanol in the microcapsules was low (0.40 % (w/w)). On the other hand, anthocyanin-rich extract/PEG particles had also an apparently amorphous morphology, indicating that anthocyanin-rich extract had been successfully encapsulated in PEG matrix via RESS using ethanol as cosolvent. The encapsulates' color was purple, confirming the presence of anthocyanin pigments in the precipitate (mass ratio between core material and PEG of 1:10), but its dispersion into the PEG matrix was not good at the operational conditions used in this study. Moreover, the anthocyanin-rich extract/PEG particles had higher agglomeration tendency than rutin/PEG particles. On the contrary, the encapsulation efficiency of anthocyanin-rich extract in PEG matrix was higher than using rutin as core material, possibly, due to its sticky characteristic. Additionally the amount of residual ethanol in these microcapsules was very similar to rutin microcapsules (0.52 % (w/w)).

RESOLV was used to obtain poly(L-lactide) (PLLA) nanoparticles loaded with retinylpalmitate (RP) (Sane and Limtrakul 2009). Retinylpalmitate and poli (L-lactide) were chosen as a liquid active compound and particle matrix, respectively. Retinylpalmitate, a vitamin A ester derivative in the retinoid group, is widely used in pharmaceutical and cosmetic applications. However, the therapeutic use of retinoids is still limited due to their light instability and adverse effects at high uptake (Cafara et al. 2006). Therefore, encapsulation is an option to protect retinylpalmitate from photo degradation induced by UVA and UVB radiation. Poly(L-lactide) has been widely used as a matrix material for drug delivery systems due to its biodegradability and biocompatibility. Three stabilizing agents, Pluronic F127, Pluronic F68, and sodium dodecyl sulfate (SDS) were tested, and Pluronic F127 was found to be more effective for stabilizing PLLA/RP nanoparticles than Pluronic F68 and SDS. 0.1 wt% Pluronic F127 solution produced a stable nanosuspension consisting mainly of well-disperse, individual nanoparticles. The effect of supersaturation, pre-expansion pressure (27.5 and 33.0 MPa), pre-expansion temperature (343.15 and 373.15 K) and concentrations of PLLA (0.1 and 0.3 wt%) and RP (0.05 and 0.15 wt%) on particle size, form, and RP loading was systematically investigated. The size of PLLA/RP nanoparticles increased from (30–80 to 30–160) nm as the solution degree of saturation changed from $S < 1$ to $S > 1$, independently of temperature, concentration of PLLA and concentration of RP. The entrapment capacity of RP in PLLA nanoparticles was predominantly determined by temperature and concentration of RP. The raise of temperature from (343.15 to 373.15) K and the concentration of RP from (0.05 to 0.15) wt% increased the encapsulated RP content at least two times. The produced PLLA/RP nanoparticles were spherical with an average size range of (40–110) nm and RP loadings of (0.9–6.2) wt%.

5.2.2 The SSI (Supercritical Solvent Impregnation) Technique

In the SSI technique, the solute is dissolved in $SCCO_2$ and put in contact with a solid matrix that must be impregnated. Ideally, the interactions created between solute and matrix are stronger than those between solute and $SCCO_2$, forcing the impregnation under supercritical conditions and followed by depressurization for the final removal of CO_2. If solute and matrix do not interact, the impregnation is achieved by depressurization of the solute-matrix- $SCCO_2$ mixture, which forces precipitation of the solute on the matrix as it is no longer soluble in CO_2. In the latter case, however, the solute is not attached to the matrix and it will be easily separated. There are different possible setups that allow different operational procedures. A convenient one is to dissolve first the solute in $SCCO_2$ until saturation, and then put it in contact with the matrix on a different reservoir. Such procedure ensures a better control of the impregnation yield. Another option is to place both solute and matrix in the same vessel from the beginning, but separated by a grid, so there is no contact between not dissolved solute and the matrix.

Figure 5.3 shows a schematic diagram of a SSI setup. The setup is basically composed of solvent cylinder, cooler, high-pressure pump, high-pressure vessel for solvation of the solute, high-pressure chamber for precipitation containing the matrix.

The parameters to be optimized are pressure and temperature during impregnation (it is related to solubility of the compound of interest and diffusion into the matrix, including possible swelling effects in the matrix), time of impregnation and rate of the depressurization step.

Applications. The SSI process was used in the impregnation of lactulose on chitosan microspheres and scaffolds (Diez-Municio et al. 2011). Lactulose is a prebiotic carbohydrate with the ability to stimulate the growth and activity of

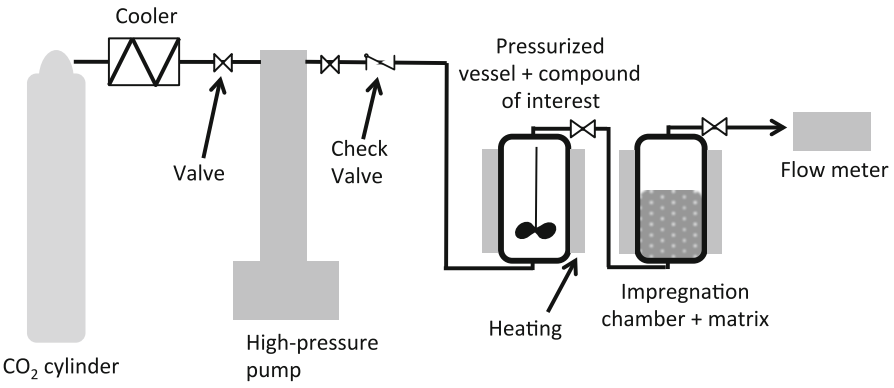

Fig. 5.3 Schematic diagram of a SSI setup

bifidobacteria and lactobacilli present in the gastrointestinal tract, performing many important functions such as protection from food allergies, regulating hormone balance, and improving the immune system (Schumann 2002). The $SCCO_2$ impregnation proved to be feasible for both chitosan forms. The highest impregnation yield (8.6 %) was obtained for chitosan scaffolds using 14 % (v/v) of cosolvent (ethanol:water 95/5, v/v) in CO_2, 10 MPa of pressure, 373.15 K of temperature, a ratio chitosan:lactulose 2:1, 60 min contact time and depressurization rate of 0.33 MPa/min.

5.3 $SCCO_2$ as Antisolvent

5.3.1 The GAS (Gas Antisolvent) and SAS (Supercritical Antisolvent) Techniques and Related Processes

The SAS technique is used when the solute is not soluble in $SCCO_2$. In this case, the solute is first dissolved in an organic solvent and then put in contact with $SCCO_2$. The organic solvent must be miscible with $SCCO_2$. As CO_2 penetrates the liquid solution, the liquid solvent loses its solvation power due to volumetric expansion of the liquid and the mixture becomes supersaturated, forcing precipitation or recrystallization of the solute. It can be visualized as $SCCO_2$ extracting the organic solvent from the liquid mixture and leaving the insoluble solute behind. The same principle applies to the GAS concept, with the only difference that the SCF is replaced by a gas. However, for mixtures where the solute interacts strongly with the solvent, volumetric expansion might not be enough to force precipitation and it is necessary to consider the vapor-liquid equilibrium of the ternary mixture.

Such observation has been exploited to develop the DELOS (depressurization of expanded liquid organic solutions) process (Ventosa et al. 2002). It is worth mentioning that CO_2 behaves in this case as a cosolvent, and therefore this technique does not fit in any of the categories described in this chapter. However, DELOS has not been applied so far in the precipitation of food ingredients and the reader is referred to Ventosa et al. (2001) for more information about the process.

The concept of supercritical antisolvent precipitation has given rise to a series of processes, each with a different acronym like SEDS (solution enhanced dispersion by supercritical fluids), ASES (aerosol solvent extraction system) and PCA (precipitation by compressed antisolvent). The differences between them are based mainly on the way that the solvent and antisolvent fluids are put in contact. The setup has implications in the particle size that can be achieved and in the possibility to carry out continuous and semi-continuous processes. However, the reader must be aware that different authors refer to either the process or the original technique to describe their work, which might bring confusion about the differences between SAS variants.

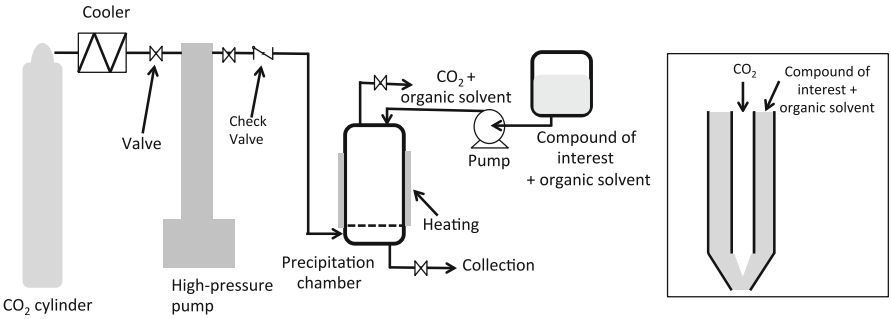

Fig. 5.4 Schematic diagram of a SAS setup. The organic solvent reservoir can be replaced by an emulsion reservoir in the case of SFEE. To the right, a detail of a two-ways coaxial nozzle commonly used for SEDS

In the most basic GAS and SAS processes, the liquid solution is initially present in the high-pressure chamber and the anti-solvent is introduced, preferably from the bottom, at the desired pressure and temperature. Precipitation occurs as the solution gets in contact with the antisolvent. After a holding time, the mixture of solvent and compressed fluid is removed from the chamber, while the particles are collected on a filter at the bottom. These processes operate in batch mode.

In the ASES (aerosol solvent extraction system) process, the high-pressure chamber initially contains the antisolvent. The liquid solution is then introduced into the chamber in form of an aerosol from the top, which causes a fast contact between solution and antisolvent, and results in smaller (micro- and nanosize) and more homogeneous particle size than in the basic SAS described above. It enables semi-continuous operation. The reader will very often find the ASES process described as SAS process in the literature.

Figure 5.4 shows a schematic setup of a SAS process. The setup is basically composed of solvent cylinder, cooler, high-pressure pumps for both CO_2 and the liquid solution (usually an ordinary HPLC pump for the liquid solution), high-pressure chamber for precipitation (with a filter to collect the produced particles at the bottom), chamber heater.

Even smaller particles can be obtained with the SEDS technique (Hanna and York 1998). In a SEDS process, a mixture of liquid solution and supercritical fluid is co-sprayed into the high-pressure chamber, which is previously filled with the supercritical fluid. The role of the anti-solvent is not only to precipitate the solute but also to mechanically disrupt the liquid solution, creating a spray of droplets smaller than those of ASES, which enables the reduction of particle size to nanoscale. The process was initially developed using a coaxial nozzle (see Fig. 5.4) to achieve intense mixing of solution and supercritical fluid before entering the precipitation chamber. But simplified versions are also possible, where premixing occurs prior to the nozzle by using a tee connection.

The PCA (precipitation by compressed antisolvent) process is recommended for a better control of the particle size and morphology in systems where a liquid-liquid phase separation may occur at high pressure (i.e. type III phase behavior in the classification by Van Konynenburg and Scott 1980). Such systems might be found when processing polymers. Even though no food applications have been found in the literature, it might be a relevant process since polymer particles can later be used as carriers for food ingredients. The PCA setup incorporates a static mixer at the top of the precipitation chamber, where the liquid-liquid phase separation occurs prior to the drying and precipitation step.

The parameters to optimize in SAS-based processes are pressure, temperature, type of organic solvent, flow rates and solvent/antisolvent ratio as well as concentration of solute in the organic solvent. For lead references on current fundamental studies of the SAS process see Rossmann et al. (2012), Tenorio et al. (2009), Martín and Cocero (2004).

Note that water solutions could in principle be dried by $SCCO_2$ using the SAS concept, if a polar organic solvent is added to the mixture.

SFEE (supercritical fluid extraction of emulsions) is a combination between particle formation with emulsions and drying of emulsions using the SAS concept. We can identify two variants of the process according to the type of emulsion (Perrut et al. 2002; Chattopadhyay et al. 2006). In water in oil (W/O) emulsions where the solute is dissolved in the aqueous phase, the supercritical fluid, even when it is $SCCO_2$, is able to remove both organic solvent and the water, as a polar organic solvent in presence of $SCCO_2$ behaves as an entraining agent of the water. If the solute is dissolved in the organic phase of an O/W emulsion, the droplets of the organic solvent become saturated by the supercritical fluid. Following the SAS principle, the solute precipitates while the supercritical fluid extracts the organic solvent. The particles are obtained as aqueous suspensions of micro- or nanosize that might need further drying depending on the application. Continuous collection is possible if a water suspension is the final product. But if the product is a dry powder it is necessary to operate in semi-continuous mode and collect the product from the precipitation chamber after depressurization.

The parameters to be optimized are those of a SAS process plus the ones affecting droplet size and stability of the initial emulsion. For emulsification techniques for food ingredients the authors refer to Ezhilarasi et al. (2013).

Applications. The SAS process has been used for particle formation of Brazilian propolis with high concentration of the bioactive compound 3,5-diprenyl-4-hydroxycinnamic acid (DHCA) (Wu et al. 2009). 3,5-diprenyl-4-hydroxycinnamic acid is the major phenolic component in propolis and possesses antioxidant, antimicrobial, anti-inflammatory, antigenotoxic, antiangiogenic and anticancer properties (Szliszka et al. 2012). The propolis extract was prepared with ethyl acetate by soxhlet. Two-factor central composite response surface methodology was used to study the effects of the concentration of propolis solution (9, 18, 27 mg/mL) and flow rate of CO_2 (10, 15, 20 L/min) on purity, recovery and mean particle size of DHCA-rich particulates. Then, pressure and temperature were set at 20 MPa and 328 K, respectively. Experimental results indicated that the purity of DHCA

increases as the CO_2 flow rate decreases. The mean particle size increases with feed concentration until 27 mg/mL. A narrow particle size distribution with mean particle size of 2 μm was obtained at high CO_2 flow rate, 20 L/min, and low feeding concentration, 9 mg/mL. Also, the DHCA concentration was increased in SAS precipitates by a factor of 1.61. The reason for narrow particle size distribution may be that a high CO_2 flow rate is associated with a faster expansion of the feed solution and high super-saturation for the nucleation of small particles. For a given degree of expansion, reducing the feed concentration promotes the supersaturation of the solute, and thus leads to narrow particle size distribution. In contrast, increasing the feeding concentration promotes the agglomeration of precipitates. In this work, the effect of the propolis particles formed by SAS on the inhibition of growth of human colon and breast cancer cells was studied. The growth of human colon and breast cancer cells that were treated with these SAS precipitated was inhibited at concentrations from (25–250) μg/mL.

Additionally, Santos and Meireles (2013) used quercetin and β-carotene as model substances in the particle formation process via SAS. Quercetin belongs to the flavonoid family and β-carotene to the carotenoids. Quercetin and β-carotene are claimed to exert many beneficial health effects. The operational conditions to carry out the particle formation of β-carotene were flow rates of CO_2 and β-carotene solution (0.8 mg/mL dissolved in dichloromethane) of 1.5 Kg/h and 1 mL/min, respectively, and the precipitation pressure and temperature were 0.8 MPa and 313.15 K, respectively. For the quercetin particle formation, the flow rates of CO_2 and quercetin solution (1.4 mg/mL dissolved in ethyl acetate) were 0.6 kg/h and 0.2 mL/min, respectively, and the precipitation pressure and temperature were 10 MPa and 313.15 K, respectively. The precipitates of quercetin obtained under the experimental conditions described above were needle-like particles with mean length of 1.872 μm. The particle size from quercetin by SAS was 4.1 times smaller than unprocessed quercetin, while conventional solvent evaporation process only reduced the particle size 1.8 times. However, a higher precipitation yield (99.5 %) was obtained using conventional solvent evaporation than using SAS (81.9 %). On the other hand, the morphology of β-carotene after SAS process, as it occurred with quercetin, also changed. The unprocessed β-carotene presented a flake-like form, while the precipitated β-carotene presented a leaf-like form. Moreover, an increase in the particle size was observed; starting from unprocessed β-carotene with mean particle size of 3.288 μm, the mean particle size of precipitated β-carotene increased to 16.090 μm. The results obtained in the work of Santos and Meireles (2013) seem to agree with the study of Franceschi et al. (2009). This work noted that most of the experimental runs produced larger particles in a wide size range dependent on the process conditions used, compared with the unprocessed β-carotene particles. In this last case, they produced β-carotene microparticles from SEDS process.

Another way to produce quercetin particles is using SEDS process (Can et al. 2009). In this study, pressure was varied between 10 and 20 MPa and temperature ranged from 313.15 to 333.15 K. The flow rates of CO_2 and quercetin solution were 10 g/min and 0.2 mL/min, respectively, at all combinations of pressure and temperature. A quercetin solution of ethyl acetate (1.4 mg/mL) was

used. The precipitates obtained under all experimental conditions were needle-like particles or flakes, with average particle size around (1–3) μm. The effect of pressure was not significant. Larger quercetin particles were obtained by increasing the temperature from (313.15 to 333.15) K. The reason may be that the supersaturation ratio is higher at higher temperature, as the CO_2 density and therefore the solute solubility decrease significantly at higher temperature. Additionally, the particle formation of quercetin by SEDS was compared with conventional crystallization with ethyl acetate. The particle size from SEDS was 6–9 times smaller than that from the conventional crystallization, which was about 9 μm. The size of quercetin particles obtained by SEDS was equivalent to that reported by Santos and Meireles (2013) using SAS.

The SEDS-PA (SEDS through prefilming atomization) process was used for the particle formation of the carotenoid astaxanthin in dichlorometane (Hong et al. 2009). Astaxanthin is a carotenoid widely used in the food, cosmetic and pharmaceutical industries as natural colorant. The particle formation of astaxanthin with different operating temperatures (308, 313, 218 and 323 K), pressures (8, 12, 16 and 20 MPa), initial solution concentrations (0.5, 1.0, 1.5 and 2.0 g/L) and solution flow (2, 3, 4 and 6 mL/min) rates was performed by the SEDS-PA process. The flow rate of CO_2 was fixed at 25 mL/min. With the increase of initial solution concentration, the particle sizes of astaxanthin microparticles decreased initially; then increased, indicating a critical concentration limit. With the increase of solution flow rate and temperature, the sizes of astaxanthin microparticles increased. With the increase of pressure, the sizes of astaxanthin microparticles decreased. Unprocessed astaxanthin crystals with particle sizes about (20–30) μm have successfully been reduced to the microparticles with sizes about (0.5–6.0) μm by SEDS-PA process.

The SAS process was applied for the particle formation of soy lecithin (Magnan et al. 2000). Soy lecithin is a mixture of phospholipids present promising therapeutic effects against dyslipidemia, atherosclerosis and cardiovascular disease (Sahebkar 2013). The result of spraying lecithin and ethyl alcohol solutions into flowing supercritical carbon dioxide was amorphous spherical and aggregated particles (from 1 to 40 μm). SAS experiments were carried out with the following experimental conditions: 308 K, precipitation temperature; (8–11) MPa, range of precipitation pressure; (2–16.5) wt%, lecithin concentration range; (10–28) mL/h, solution flow rate range; and 400 g/h CO_2 flow rate. The increase of the solute concentration and solution flow rate had a marked influence upon the particle size, while pressure did not seem to have any effect. Two opposite effects depending on the liquid flow rate were observed. For instance, at 10 mL/h, an increase of the concentration yields and increase of the particle size, while at 28 mL/h the opposite effect was observed.

SAS technique, analogically to the RESS technique, was employed for encapsulating bioactive compounds. For instance, Heyang et al. (2009) encapsulated lutein in hydroxyl propyl methyl cellulose phthalate (HPMCP) to maintain its bioactivity and avoid thermal and light degradation. Lutein is a carotenoid with high antioxidant capacity and many beneficial health effects (Koushan et al. 2013).

In this work, the effects of flow rate of CO_2 (0.88, 1.50 and 2.00 Kg/h), pressure (11, 13 and 15 MPa), temperature (313.15, 318.15 and 323.15 K), concentration of HPMCP (1, 2, 4 and 8 mg/mL) and lutein (0.1 and 0.8 mg/mL) and the ratio of HPMCP/lutein (10:1, 10:2 and 10:4) were studied. Many operating parameters affected the yield, such as lutein loading, encapsulation efficiency, particle size, and distribution of the nanocapsule. The authors reported that the mean diameter of a lutein-loaded HPMCP nanocapsule was in the range from (163 to 219) nm. The highest encapsulation efficiency for lutein (88 %) and recovery yield (16 %) were obtained under operating conditions of 11 MPa at 313.15 K with 10:4 ratio of HPMCP and lutein.

Martín et al. (2007) developed a semicontinuous supercritical antisolvent (SAS) process for the production of a mixture of β-carotene or lutein and poly-ethylene glycol (PEG) by co-precipitation. PEGs are water-soluble polymers that are widely used in pharmaceutical and cosmetic industries because of their physiological acceptance. The co-precipitation of β-carotene and PEG was carried out with an operating temperature of 288 K and an operating pressure of 8 MPa. They tested the influence of different PEG initial concentrations (12 and 16 g/L) and CO_2 flow rates (2.5 and 3.5 kg/L). Prismatic particles of β-carotene of (100–200) μm were produced. The carotenoid particles were only partially covered with PEG. Furthermore, little or no effect of the process parameters on product characteristics was found in the range of conditions considered in this work, as all the experiments yielded very similar results. In the case of lutein, the product obtained consisted of spheres of PEG of about 50 μm of diameter, which covered the lutein almost completely. These particles had a spherical and apparently amorphous morphology. The temperature was set up at 288 K in all experiments. The influence of CO_2 flow rate (2.5 and 3.5 Kg/L) was studied. With lower CO_2 flow rates, more agglomerated lutein particles, which were less covered by PEG. A variation of pressure in the range 8–10 MPa had a very small effect on both morphology and size of the particles. The initial concentration of lutein (1–3 g/L) was varied. A decrease in PEG/lutein ratio led to the production of agglomerated prismatic particles of lutein. The smaller size of lutein particles compared to the β-carotene particles may be the reason why it has been possible to produce well coated particles of lutein, but not of β-carotene.

Bixin is a carotenoid very sensitive to degradation. Thus, a bixin-rich extract was encapsulated with polyethylene glycol (PEG) 10,000 through the SAS process (Santos and Meireles 2013). The solvent selected was dichloromethane because it is a good solvent for both the bixin-rich extract and PEG. The solution flow rate, precipitation pressure and temperature were fixed at 1 mL/min, 10 MPa and 313.15 K, respectively. The CO_2 flow rate and mass ratio between bixin-rich extract and PEG investigated were (0.6 and 1.5) kg/h, 1:2 and 1:10, respectively. Using the lowest CO_2 flow rate, the loss of bixin-rich extract encapsulated was reduced; thus, this flow rate was selected for further experiments. Regarding the effect of the ratio between bixin rich-extract and PEG, a decrease in the mass ratio between core material and encapsulating material, i.e., and increase in the polymer concentration, led to the production of less agglomerated particles. The smaller mass ratio (1:10)

between bixin-rich extract and PEG was chosen, because for the mass ratio of 1:2, it was visually observed that the amount of polymer was not sufficient to effectively encapsulate the amount of bixin-rich extract. The morphology of the microparticles of bixin-rich extract encapsulated in PEG was diverse, such as flake-like, bar-like, etc. Bixin-rich extract/PEG particle size of 33 μm was produced.

The SAS process was used in a food grade polymer-corn zein, a category of alcohol-soluble proteins, as the carrier material for microencapsulating bioactives (Zhong et al. 2008). Zein is insoluble in aqueous solutions; zein-based delivery systems may thus maintain the integrity in aqueous food products during processing and storage. Three alcohols, i.e. ethanol, methanol, and isopropanol, with appropriate amount of water were used to dissolve zein. The SAS process was applied to synthesize micro- and nanoparticles of zein for edible delivery systems of bioactive compounds. In this work, different critical variables were studied, such as polymer concentration (0.5–10 % w/v), CO_2 flow rate (10–100 g/min), and solvent chemistry (85 and 90 % of ethanol; 90, 95, 98 and 100 % of methanol; and 90 % of isopropanol). The operation conditions were set up at 313.15 K and 10 MPa. In the case of 1.0 % starting concentration of zein, precipitation was only achieved when 100 % methanol was used as solvent. The best CO_2 flow rate was 100 g/min. The nanoparticles obtained have an average diameter of (105.5 and 79) nm, for the (1.0 and 0.5) % initial concentration of zein, respectively. Accordingly the manipulation of the above variables enabled the production of micro- and nanoparticles, which can be used as bases for microencapsulating bioactives.

5.4 $SCCO_2$ as Dispersant or Solute

5.4.1 PGSS™ (Particles from Gas-Saturated Solutions) Technique and Related Processes

PGSS™ is the technique chosen when the compound of interest is not quite soluble in the SCF but it is able to absorb high amounts of it. In a PGSS™ process, the compound of interest is a solid that melts when pressurized by compressed or $SCCO_2$ in a static mixer or autoclave. In this case, $SCCO_2$ is the solute and it creates a gas-saturated solution of the melt. The saturated melt is rapidly expanded through a nozzle to atmospheric pressure. The fast depressurization causes volumetric expansion of the gas, which disrupts the melt into small droplets. At the same time, the intense cooling created by the Joule-Thomson effect induces solidification of the sample. The PGSS™ technique can also be applied to liquid solutions or suspensions, but these cases might require further drying. The parameters to optimize are the pre-expansion pressure and temperature.

Figure 5.5 shows a scheme of a PGSS™ setup. The setup is basically composed of solvent cylinder, cooler, high-pressure pump, autoclave or static mixer and expansion chamber for precipitation.

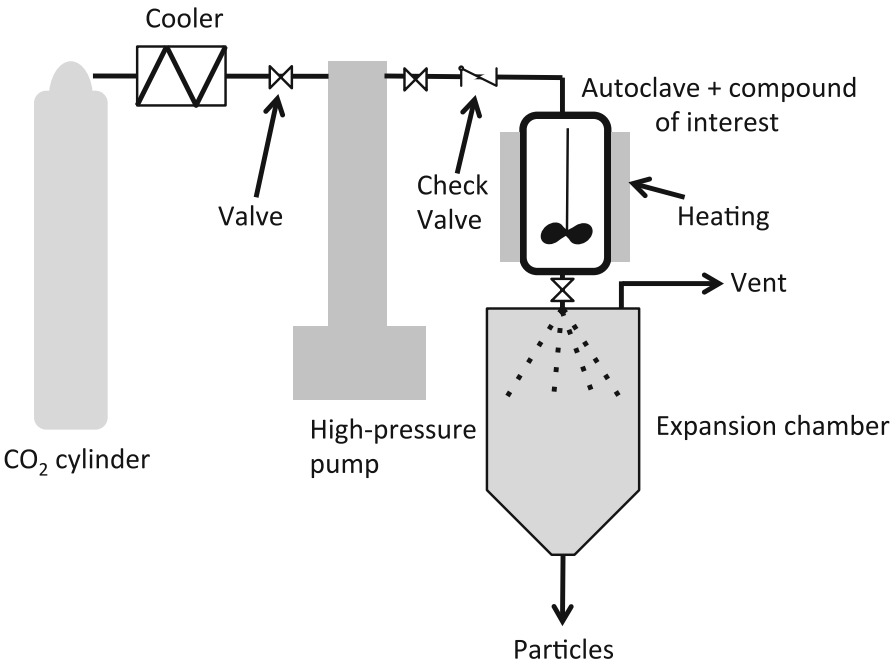

Fig. 5.5 Schematic diagram of a PGSS™ setup

PGSS™ requires lower pressures than RESS or SAS processes and lower gas consumption, it is easier to scale up and suitable for viscous samples. For further details about the technique, the authors refer to Knez and Weidner (2003) and Strumendo et al. (2007).

Processes like ScMM (supercritical melt micronization) and CAN-BD® (carbon dioxide assisted nebulization with a bubble dryer) are based on the PGSS™ technique. The ScMM is a process based on the PGSS™ principle and applied to the particle formation of hard fats. The CAN-BD® process was developed to dry aqueous solutions (Sievers and Karst 1995). In the CAN-BD® process pressurized CO_2 is put in contact with an aqueous solution for a brief time to form an emulsion. The emulsion passes through a restrictor into a warm and atmospheric pressure container and forms an aerosol, as CO_2 expands during decompression. The aerosol is usually dried by a current of hot nitrogen gas in the expansion container. Instead of the static mixer used in the PGSS™, there is a low dead-volume tee connection where the liquid solution and the CO_2 get in contact. Unlike in PGSS™, the liquid solution is not saturated by CO_2 but creates the emulsion that will form an aerosol. For this reason, CAN-BD® could be considered as a particle formation technique on its own. The parameters to be optimized are the concentration of the compound of interest in water, as well as those affecting the characteristics of the aerosol (pressure, temperature, flow rate and restrictor geometry).

Applications. The PGSS™ technique has been used mainly for the encapsulation of bioactive compounds. For instance, De Paz et al. (2012) encapsulated β-carotene into soybean lecithin using the PGSS-drying technique. The application of β-carotene as a natural colorant in food and nutraceutical products requires an appropriate formulation in order to protect the active compound from degradation and overcome the low bioavailability due to a low solubility in aqueous media. The authors studied the influence of pressure, temperature, gas to product ratio and concentration of carrier material in the particle size and encapsulation efficiency. An aqueous suspension of β-carotene and lecithin was processed using PGSS™ as drying technique in order to remove water and precipitate the lecithin dissolved over the preformed β-carotene particles. Temperature varied from (373.15 to 403.15) K and pressure was modified from (8 to 10) MPa. Also the suspension flow rate was varied between (21 and 37) g/h and the experiment ran for 60 min. The encapsulation efficiency of β-carotene increased with pre-expansion temperature, up to 60 %. Dry spherical particles were obtained with sizes ranging from 10 to 500 μm.

The PGSS™ technique has also been used to encapsulate probiotics, which are defined as "the living beneficial bacteria that support digestion as well as vaginal and urinary tract health" (Vermuri et al. 2014). Probiotics promote the body's natural immunity and keep us healthy. These probiotics are well exploited in food, medical and aquaculture industries. Moolman et al. (2006) carried out encapsulation of probiotics with an interpolymer complex by the PGSS™ technique. Bifidobacteria exhibit limited survival in environments with oxygen, and they are sensitive to temperature and solvents. PGSS™ as encapsulation technique avoids such exposure during the process. The method was used to encapsulate indomethacin and *Bifidobacterium longum* in a poly(vinyl pyrrolidone)-poly(vinyl acetate-co-crotonic acid) interpolymer complex (PVP:PVAc-CA). The conditions were 30.0 MPa of CO_2 pressure, 313.15 K of temperature and 2 h of equilibrating time. After this, the plasticized product was sprayed into a 10 L expansion chamber pressure-controlled at 1.5 MPa. The plasticization induced by the PGSS™ process originated porous polymer particles. The mean particle size was 168 μm. Particle size was reduced by more than an order of magnitude (mean diameter 6.9 μm) through the addition of a suitable viscosity modifier, i.e. glycerylmonostearate. It was shown that the encapsulation matrix is stable at low pH, but disintegrates at higher pH, triggering the release of the encapsulated material. In a later work (Mamvura et al. 2011), the same group reported the encapsulation of other probiotic strain, *Bifidobacterium lactis*, using the same polymers and the same operation conditions of PGSS™. The average particle size of PVP:PVAc-CA interpolymer complex matrix microparticles encapsulating *B. lactis* Bb12 was 166.1 μm. An average encapsulation efficiency of 96 % was reported. Consequently, the microparticles have the potential to be evenly distributed in foods, deliver adequate amounts of probiotic and produce minimal adverse effects on the texture and mouth feel of the foods where they are incorporated.

Furthermore, Lubary et al. (2011) performed the formation of microparticles from anhydrous milk fat (AMF) and a diacylglycerol-based modified milk fat (D-AMF) by ScMM. ScMM experiments were carried out varying the temperature of the melt (306–334 K) and the dissolution pressure (7–20 MPa). Both fats were able to dissolve 30 wt% of CO_2 in the studied pressure and temperature ranges, being the CO_2 amount slightly higher for AMF. A melting point depression was observed in both systems in the presence of CO_2. Two different particle morphologies, spherical hollow and sponge-like particles, in an approximate size range (10–40) μm were obtained, depending on the CO_2 concentration in the fat melt in the first place, and on the pre-expansion temperature of the melt in the second place. Small broken particles originated from the breakage of spherical fat particles that solidified before all CO_2 could escape from the atomized droplets. While the hollow spheres had a tendency to agglomerate, the broken microparticles formed a free-flowing powder as long as they were stored at low temperatures. Sponge-like particles, despite their agglomeration appearance, were free-flowing at low temperature (255.15 K) and had lower bulk density than hollow spheres, which showed a certain trend to agglomerate. Both types of particles have potential for being incorporated in refrigerated or frozen food products as a structuring agent.

5.5 Hyphenated Techniques

In the last decades, food science and technology have benefited from the use of advanced extraction techniques like pressurized fluid extraction (PFE) to obtain food ingredients from natural sources. Removal of extraction solvents has been a challenge that limits their application in food industry. To overcome this problem, in the last few years there has been a tendency to hyphenate PFE with particle formation techniques based on SCFs as a drying procedure. The WEPO and OEPO processes are examples of such hyphenations.

5.5.1 WEPO (Pressurized Hot Water Extraction and Particle Formation On-Line)

WEPO is a process that combines pressurized hot water extraction (PHWE) with particle formation in one-step. The WEPO process uses the CAN-BD® approach for the drying of a warm aqueous solution that comes directly from a continuous extraction process (PHWE) (see Fig. 5.6). PHWE is a technique that uses water at high temperature (usually above its boiling point) and pressure to keep it in liquid state. The aqueous solution in WEPO contains more than one compound and they all precipitate during the drying step. The concentration and composition of the aqueous solution affect the particle size of the final product, and are influenced by

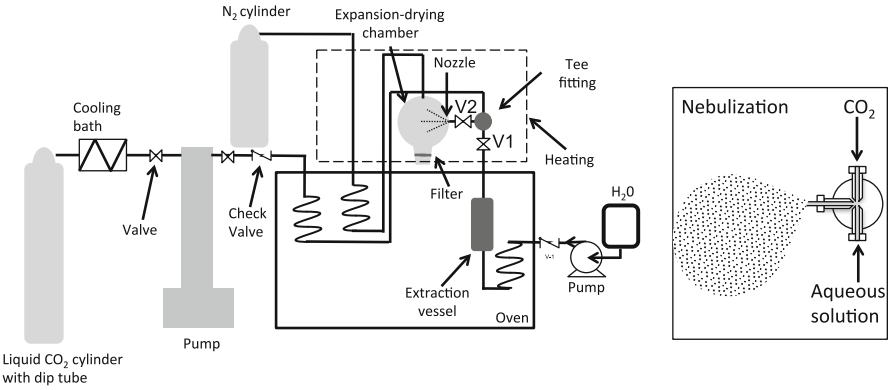

Fig. 5.6 Scheme of the WEPO process. To the right, detail of the nebulization through a CAN-BD-type tee connection

the extraction parameters such as temperature and flow rate. At the same time, temperature and flow rate, together with pressure and restrictor geometry, affect the aerosol formation. Therefore, process optimization might require compromises between an optimal extraction and an optimal particle formation process.

5.5.2 *OEPO (Organic Solvent Extraction and On-Line Particle Formation)*

The concept of the WEPO process has been used by Santos and Meireles (2013) to develop the OEPO process, in which pressurized fluid extraction (PFE) with organic solvents is combined with particle formation via the SAS technique in its different variants. The extract from PFE is a liquid solution containing more than one compound of interest. It can be mixed with a second solution of a carrier or a surfactant prior to antisolvent precipitation, respectively leading to a co-precipitation via SAS or encapsulation via SFEE. Unlike the WEPO process, it is very likely that not all the extracted compounds will precipitate upon contact with the antisolvent during OEPO. Precipitation of each compound will depend on the parameters discussed for the SAS technique (see Sect. 5.3), with the added difficulty of possible interactions between the different compounds extracted and even more complex phase behaviors when the carrier/surfactant is dissolved in a different solvent than the extract.

Applications. The WEPO process has been employed by Rodríguez-Meizoso et al. (2012) and Herrero et al. (2010) to obtain bioactive compounds from rosemary leaves. This plant has been widely studied due to the strong antioxidant capacities associated to some of its components; among them, phenolic diterpenes have attracted more attention (Wellwood and Cole 2004). Based on previous works, water extraction at 473.15 K was selected to achieve the maximum antioxidant

capacity, while water flow rate was study to determine its influence on powder formation. Other parameters influencing the drying process, such as CO_2 pressure (8.0 MPa) and flow rate (2.5 mL/min) and N_2 flow rate (0.6 mL/min) were settled to obtain a fine and constant spray. They carried out the experiments with three different water flow rates, (0.1, 0.2, and 0.3) mL/min, respectively. The best flow rate for obtaining smaller particles size and higher yields was 0.2 mL/min. The authors collected microparticles that precipitate in agglomerates formed by smaller particles with no defined size as a fine powder with diameters lower than 93 μm. Other application of WEPO process was developed by Andersson et al. (2012). They used WEPO process for getting dried extract from onion with the same composition of quercetin derivatives as non-dried extracts. They obtained a fine powder with spherical particles from 250 nm to 4 μm in diameter. The optimal parameters, such as temperature (293.15 K), $SCCO_2$ and N_2 pressures (8.0 and 1.3 MPa, respectively) and flow rate of $SCCO_2$ (10 mL/min), were settled by trial-and error in order to achieve a fine and constant spray formation. The WEPO process described in these works can be considered as a suitable and promising process to obtain, in a unique step, a fine dried powder with intact antioxidant capacity, directly from plants (Ibanez et al. 2009).

The OEPO process has been applied to obtain antioxidant products from Brazilian ginseng roots (*Pfaffia glomerata*) using ethyl acetate as extracting solvent. The process conditions reported were a 15 min static PFE extraction at 373 K and 12 MPa, followed by dynamic extraction with a solvent to feed volume ratio of 20 cm^3 of solvent/4.5 g of milled roots. CO_2 was delivered at 313 K and 10 MPa, and constant flow rate of 0.6 kg/h. In the case of combining the ethyl acetate extract with an extra flow of surfactant/carrier solution, the total flow rate of liquids was 1.0 cm^3/min. The suspensions obtained by PFE-SFEE were further freeze-dried to produce a dry powder. The products were analyzed in terms of yield (dry basis) and antioxidant activity and compared to those obtained when performing PFE and SAS-precipitation in different steps. A loss of 14 % antioxidant activity during OEPO respect to the two-steps procedure was reported. The co-precipitated particles showed irregular shapes on the micro-size (up to 50 μm length in the reported images), while the encapsulated particles showed spherical shapes with smooth surfaces also in the micro-size range.

Conclusions

The application of well-known particle formation techniques based on supercritical fluids into the processing of food ingredients has not been extensively explored so far, in comparison to the high amount of publications related to the processing of drugs, explosives and inorganic materials.

In the case of food ingredients, process development has been mainly focused on observing particle size and morphology, while claimed particle properties like improvement of solubility in water, stability towards oxygen

(continued)

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and heat, and bioavailability are not deeply studied. It has been observed by the authors that the majority of research articles on the topic involve mainly pure food ingredients from chemical synthesis (standards). However, the acceptance of food ingredients by consumers is higher when those chemicals come from natural sources. It is expected that such natural ingredients are associated with more complex samples (e.g. with more than one compound), as they are obtained from more or less selective extraction procedures. In this sense, the complexity of a particle formation process will only increase and process optimization will just become more difficult by any kind of predictive tool. Therefore, we do not expect process optimization to move away from the trial-and-error approach, but rather an improvement in experimental design including possible interactions between different process parameters.

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Chapter 6
Enzymatic Reactions in Supercritical Fluids

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6.1 Introduction

Biotransformations have been of tremendous social and economic importance throughout the history of mankind. Beer and bread production dates back 6000 years B.C., while vinegar production dates back to some 2000 years B.C. Production of organic chemicals by biotransformations started in the second half of nineteenth century (Liese et al. 2000).

Nowadays several pharmaceuticals, amino acids, saccharides and polysaccharides, esters and vitamins are produced by enzymatic biotransformations already on industrial scale (Liese et al. 2000). All these reactions are performed either in water or seldom in organic solvent as reaction media.

Enzymatic catalysis has gained considerable attention in recent years as an efficient tool for synthesis of natural products, pharmaceuticals, fine chemicals and food ingredients.

The production of fine chemicals results in the generation of considerable volumes of waste, as the syntheses generally include a number of steps. The yield of each of these steps is usually (60–90) %, but 10 % is not unusual. Based on these data we can conclude that typically 1 kg of end-product leads to the generation of 15 kg of wastes or more. Most of the wastes generated are solvents and by-products from solvents and intermediates. Therefore, ideally, several reactions should be performed either in water or in supercritical fluids (SCFs).

Nowadays SCFs may be an alternative to conventional solvents. Reactions of polymer synthesis and hydrogenation in subcritical propane or hydrogenation in supercritical carbon dioxide, oxidation reactions in supercritical water are applied on large-scale production in the twentieth century. Use of supercritical solvents for

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synthesis of complex organic molecules is under development. Research activities on application of SCFs as reaction media for biocatalytic reactions is well documented in several reviews (Habulin et al. 2007; Hobbs and Thomas 2007; Rezaei et al. 2007; Sheldon 2005; Krieger et al. 2004; Krishna 2002; Knez and Habulin 2002; Knez et al. 2005; Ikushima 1997; Halling 1994; Nakamura 1990).

The first reports on the use of SCFs as reaction media for enzyme transformations are dated back in 1985 (Randolph et al. 1985). Since then several publications on oxidation (Randolph et al. 1988a, b; Findrik et al. 2005), hydrolysis (Glowacz et al. 1996; Rantakylä et al. 1996; Perve et al. 1997; Holmes et al. 1998; Hampson and Foglia 1999; Rezaei and Temelli 2000, 2001; Hartmann et al. 2001; Turner et al. 2001a, b; Park et al. 2001; Habulin and Knez 2001a, 2002; Martinez et al. 2002; Nakaya et al. 2002; Hakoda et al. 2003; Knez et al. 2003; Primožič et al. 2003, 2006; Sovová and Zarevućka 2003; Habulin et al. 2005a, b; Muratov et al. 2005; Guthalugu et al. 2006; Bártlová et al. 2006; Salgin et al. 2007; Sovová et al. 2008), transesterification (Vermue et al. 1992; Kamat et al. 1993; Madras et al. 2004a; Weber et al. 2008), esterification (Lin et al. 2006; Varma and Madras 2007; Šabeder et al. 2005; Romero et al. 2005; Nagesha et al. 2004; Madras et al. 2004b; Matsuda et al. 2004; Kumar et al. 2004; Srivastava et al. 2003; Rezaei and Temelli 2000; Catoni et al. 1996; Marty et al. 1994) and enantioselective synthesis (Matsuda et al. 2001a, 2004; Palocci et al. 2008; Salgin et al. 2007; Nakamura et al. 2003; Ottosson et al. 2002; Hartmann et al. 2001; Celia et al. 1999; Capewell et al. 1996; Cernia et al. 1994) have proved the feasibility of enzymatic reactions in SCFs. The temperature ranges used for employing dense gases in processing are compatible with the use of enzymes as catalysts. However, a limitation of the process may arise from the non-polarity of carbon dioxide (CO_2), which preferentially dissolves hydrophobic compounds. However, recent advances in the understanding of the chemical properties of materials that are soluble in CO_2 have permitted the development of novel surfactants that allow dissolution of both hydrophilic and hydrophobic materials in CO_2. This has made it possible to consider the use of CO_2 as a solvent in a wide variety of manufacturing processes. On the other hand, several other "non-green" gases (fluorocarbons, propane, butane, dimethyl ether, SF_6) have been used recently (Habulin et al. 2005a, b; Knez et al. 1995, 1998, 2003, 2005; Novak et al. 2003; Habulin and Knez 2001b; Krmelj et al. 1999; Saul et al. 2004).

The high selectivity and mild reaction conditions of enzymatic transformations make them an alternative to the synthesis of complex bioactive compounds, which are often difficult to obtain by standard chemical routes. However, the majority of organic compounds are not highly soluble in water, which was traditionally perceived as the only suitable reaction medium for the application of biocatalysts. The realization that most enzymes can function perfectly well under nearly anhydrous conditions and, in addition, display a number of useful properties, e.g., highly enhanced stability and different selectivity, has dramatically widened the scope of their application to organic synthesis. Another great attraction of using organic solvents rather than water as a reaction solvent is the ability to perform synthetic transformations with relatively inexpensive hydrolytic enzymes. Generally, *in vivo*,

the synthetic and hydrolytic pathways are catalyzed by different enzymes. However, elimination of water from the reaction mixture enables the "reversal" of hydrolytic enzymes and thus avoids the use of the expensive cofactors or activated substrates that are required for their synthetic counterparts. Water is the most common solvent for biochemical reactions. Still, in a biotechnological perspective, there are a lot of advantages of conducting enzymatic conversions in monophasic organic solvents as opposed to water (Dordick 1989), as listed below:

- High solubility of most organic (non polar) compounds in non aqueous media
- Ability to carry out new reactions impossible in water because of kinetic or thermodynamic restrictions
- Reduction of water-dependent side reactions
- Insolubility of enzymes in organic media, which allows their easy recovery and reuse

However, the use of solvents can be problematic because of their toxicity and flammability, and also because of increasing environmental concerns. As a result, SCFs have attracted much attention in recent years as an alternative to organic solvents for enzymatic reactions.

The use of SCFs as solvents for enzymatic transformations is a relatively new area of research, which is expected to expand in the future. Our Chapter will focus on the development of selective methods for the production of polyfunctional molecules by enzymatic reactions in supercritical carbon dioxide ($SCCO_2$).

Among all possible SCFs, CO_2 is the most widely used. The use of $SCCO_2$ instead of organic solvents in biocatalysis presents several advantages: its critical pressure (7.38 MPa) is "acceptable"; its critical temperature (31.1 °C) is consistent with the use of enzymes and/or labile solutes and it has the GRAS (Generally Regarded As Safe) status. In addition, its "naturalness" is greatly appreciated by the food and health-care related industries.

Close to the critical point, small changes in temperature or pressure can produce large changes of density and solvation ability of SCFs. Beyond the critical point, both phases are indistinguishable and the fluid is monophasic and occupies all the vessel volume. It can be described as a dense gas or an expanded liquid. Generally, SCFs exhibit liquid-like density and therefore have good solvating power, but they retain gas-like compressibility. Consequently, it is possible to control their solvating power by changing the pressure and/or temperature, with a continuous transition from a good to poor solvent. Moreover, low viscosity and high diffusion coefficients of these fluids enhance mass transport and reaction kinetics. These unique properties of SCFs enable one to design efficient integrated processes by coupling an enzymatic reaction with subsequent fractionation and product recovery steps.

Molecules in the SC phase are not uniformly distributed in space, but the solvent molecules aggregates around the solute through solvent-solute intermolecular interactions forming clusters, where the aggregated molecules are in dynamic equilibrium with free solvent molecules. Thus, the solvation depends strongly on the density of the SCFs and differs from that in the liquid solution or gas phase

(Kajimoto 1999). When catalytic reactions are performed in SCFs, the outcome of the reactions can be affected in a number of ways. In general, replacement of conventional liquid solvents by SCFs can increase the rate and tune the selectivity of reactions for the following reasons (Ikariya and Kayaki 2000):

- Rapid diffusion of solutes or weakening of the solvation around reacting species facilitates the reactions and sometimes changes the reaction pathway
- Local clustering of solutes or solvents resulting in an appreciable increase in the local concentration of substrate (and catalyst) causes acceleration of the reaction
- Reduction and/or increase in the cage effect often affects the reaction performance of rapid chemical transformations such as radical reactions

The benefits of SCFs as reaction media include economical, technical, environmental, and health advantages. The environmental benefits of the application of SCFs in industrial processes are a result of the replacement of environmentally far more damaging conventional organic solvents. Another environmental impact is the low energy consumption during operation. Health and safety benefits arise from the fact that the most important SCFs ($SCCO_2$ and SC H_2O) are non-carcinogenic, non-toxic, non-mutagenic, non-flammable and thermodynamically stable.

The high volatility of CO_2 allows it to be completely and easily removed from the product, resulting in an overall "solvent-free" reaction. By using $SCCO_2$ an integrated production process can be performed, because it can act as a solvent for the reaction and also as a separation medium. The variable solvating power of $SCCO_2$ (and other SCFs) facilitates the integration of biocatalytic and downstream processing steps in a single robust bioreactor.

The main drawback of $SCCO_2$ is that it has limited solvating power with respect to polar compounds. This is a serious limitation for biotechnological applications where most natural molecules of interest (e.g., alkaloids, carotenoids, phenols, proteins, and sugars) are only sparingly soluble in $SCCO_2$. In this case, a polar cosolvent or a so-called "entrainer", such as acetone, ethanol, methanol, or water is added in order to increase the polarity of the medium and to solubilize the target solute via the formation of hydrogen bonds. Typically, cosolvents are added to the SCF at moderate concentrations of less than 10 mol % (Wong and Johnston 1986). In a batch reactor, the cosolvent can be added directly into the reactor prior to pressurization, whereas in a continuous process, the addition should be made to the CO_2 inflow via a liquid pump to deliver a constant flow rate at the operating pressure. However, the use of another component in the system further increases the complexity and may also complicate downstream processing. Moreover, the solubility enhancement effect of a co-solvent is usually limited in the case of very polar compounds.

Two alternative methods have been developed for some specific cases. To solubilize polyols (e.g., glycerol and sugars) it has been proposed to form a hydrophobic complex between the polyol and phenylboronic acid (PBAC), which is much more soluble in the SC phase (Castillo et al. 1994). This method was used to perform esterification of glycerol and sugar with oleic acid in $SCCO_2$. The other method involves the adsorption of polar substrates onto a solid hydrophilic support

such as silica gel. Compared with the former, this approach is more general because it is not necessary to have two vicinal hydroxyl groups in the substrate molecule (Castillo et al. 1994). In addition, recent advances in the understanding of the chemical properties of materials that are soluble in CO_2 have permitted the development of novel surfactants that allow dissolution of both hydrophilic and hydrophobic materials in CO_2.

The use of SCFs decreases the mass transfer limitations because of the high diffusivity of reactants in the SC medium, the low surface tension, and because of the relatively low viscosity of the mixture. The Schmidt number, $Sc = \eta/\rho \cdot D$, (where η is dynamic viscosity, D is diffusivity and ρ is density) for CO_2 at 200 bar is 45 times lower than that for water at 1 bar and 20 °C. High diffusivity of SCF and low surface tension lead to reduced internal mass transfer limitations for heterogeneous chemical or biochemical catalysis.

One of the main advantages of the use of dense gases as a solvent for enzyme-catalyzed reactions is the simple downstream processing. The physico-chemical properties of dense gases are determined by their pressure and temperature, and are especially sensitive near their critical point. By reducing the solvent-power of a dense gas in several stages, fractionation of the product and unreacted reactants is possible. Fractionation is also possible by extracting the mixture, usually with the same dense gas as used in reaction, but under different process conditions. In all downstream processing schemes, various particle-formation techniques or chromatographic techniques can be integrated.

Usually high-pressure batch reactors are used for the screening of the enzymes and for determination of kinetics of enzymatic reactions. Some reactions are also performed in continuous reactors. Among these, continuous packed bed reactors have been used for immobilized enzymes. Recently, high-pressure membrane reactors with bio-active membranes are used.

6.2 Enzymatic Catalysis in Supercritical Fluids

Modern biocatalysis is developing new and precise tools to improve a wide range of production processes, which reduce energy and raw material consumption and generate less waste and toxic side-products.

The growing interest in industrial biocatalysis and recent scientific advances in enzymatic catalysis opened a wide range of applications in the fields of pharmaceuticals, fine chemicals, intermediates etc., leading to an increasing number of industrial biotransformations.

First reports on biocatalysis in non-aqueous media are dated in the early 1980's (Dordick 1989; Kajimoto 1999; Wong and Johnston 1986; Castillo et al. 1994; Antonini et al. 1981; Martinek et al. 1981; Zaks and Klibanov 1984, 1985).

Nowadays, it is well established that many enzymes can remain active and stable in pure organic solvents. A change from an aqueous environment can favor a shift in the equilibrium enabling synthetic reactions to be achieved with hydrolytic

enzymes. Enzyme inactivation, caused by hydrolysis of peptide bonds and deamidation of asparagine and glutamine residues, could be reduced using media of low water content (Klibanov 2001). Indeed, many times enzymes are more stable in organic solvents than in water.

Many substrates that are insoluble in water can be dissolved by organic solvents. From economical point of view, the insolubility of enzymes in organic solvents presents an advantage that simplifies its recovery and reuse. Furthermore, enzymes are environmentally benign and are, unlike metal catalysts, completely degraded in nature.

Numerous studies on enzyme activities in non-aqueous media, including pure organic solvents and SCFs, contributed to discard the notion that enzymes are only active in aqueous media. First experiments using enzymes in non-aqueous media date back to the end of the nineteenth century (Hill 1898; Kastle and Loevenhart 1900; Bourquelot and Bridel 1913; Dastoli and Price 1967).

Enzymatic reactions in no aqueous media offer new possibilities for producing useful chemicals e.g. surfactants, emulsifiers, wax and flavor esters (Krishna and Karanth 2001, 2002; Krishna et al. 1999, 2000a, b, 2001a, b), high-value pharmaceutical substances (Zaks and Dodds 1997; Schulze et al. 1998; McCoy 1999; Patel 2001; Rasor and Voss 2001), chiral drug molecules (Klibanov 1990; Collins et al. 1992; Stinson 2000; Zaks 2001), biopolymers (Kobayashi 1999), peptides, proteins and sugar-based polymers (Vulfson 1998), modified fats and oils (Bornscheuer 2000), structured lipids etc.

In no aqueous solvents, hydrolytic enzymes could undergo synthetic reactions while they also exhibit altered selectivities (Klibanov 2001), pH memory (Zaks and Klibanov 1985, 1988a; Klibanov 1995), increased activity and stability at elevated temperatures (Zaks and Klibanov 1984; Ahern and Klibanov 1985), regio-, enantio- and stereoselectivity (Bornscheuer 2000) and may also be affected by their water activity (Halling 2000). Currently, there is a considerable interest in the use of enzymes as catalysts in organic synthesis (Faber 2000; Bornscheuer 2000; Schmid and Verger 1998; Carrea and Riva 2000; Liese et al. 2000; Patel 2000; Koeller and Wong 2001).

Five major technological advances are believed to have significantly influenced the industry for adopting enzymatic biotransformations (Lilly and Eighth 1994): (1) the development of large-scale downstream processing techniques for the release of intracellular enzymes from microorganisms; (2) improved screening methods for novel biocatalysts (Kieslich et al. 1998; Demirjan et al. 1999; Wahler and Reymond 2001; Asano 2002; Ornstein 2002); (3) the development of immobilized enzymes; (4) biocatalysis in organic media; and most recently (5) recombinant-DNA technology to produce enzymes at a reasonable cost. The establishment of industrial processes (Coleman and Macrae 1977; Matsuo et al. 1981), and the realization that most enzymes can function well in organic solvents (Zaks and Klibanov 1984, 1985, 1986, 1988b) have heightened interest in the use of enzymes. Also, the need for enantiomerically pure drugs is driving the demand for enzymatic processes. This, combined with the discovery of strikingly

new properties of enzymes in organic solvents, has led to establishment of organic phase enzyme processes in industry (Bornscheuer 2000; Liese et al. 2000).

Enzymes have a unique place in synthetic chemistry due to their high selectivities and rapid catalysis under ambient reaction conditions. The fact that enzymes are stable, and in some cases, improve their high specificity in near anhydrous media, has dramatically changed the prospects of employing enzymes in synthetic organic chemistry. The problems that arise for most biotransformations are low solubility of reactants and products, and limited stability of biocatalysts. Carrying out reactions in an aqueous-organic two-phase system would be a solution to overcome the first problem. This is not always possible due to the limited stability of enzymes at liquid–liquid interface or in organic solvents.

Solvent systems used as reaction media for enzymatic catalysis may be categorized as: (1) aqueous; (2) water: water-miscible (monophasic aqueous-organic system); (3) water: water-immiscible (biphasic aqueous-organic system); (4) non aqueous (monophasic organic system); (5) anhydrous; (6) SCFs; (7) reversed micelles; (8) solvent-free systems; (9) gas phase; and (10) ionic liquids.

The interest of using biocatalysts in $SCCO_2$ has been growing rapidly in recent years, mainly in industrial and pilot plant applications (Perrut 2000). Several enzymatic reactions, such as oxidation, hydrolysis, transesterification, esterification, enantioselective synthesis were performed in SCFs.

Advantages of using $SCCO_2$ as a medium for enzymatic catalyzed reactions have been well documented (Aaltonen and Rantakyla 1991; Nakamura 1994; Knez et al. 1998). In addition, its "naturalness" is greatly appreciated by the food and health-care related industries. Its capacity of encouraging transport phenomena (due to high diffusivities) and facilitating reaction products separation by tuning solvent power makes $SCCO_2$, extremely attractive to use as 'green-designer' solvent for environmentally more acceptable chemical processes (Jarzebski and Malinowski 1995; Blanchard et al. 1999; Lozano et al. 2002, 2004; Hitzler et al. 1998). However, it has also disadvantages, as, for example, lower catalytic activities in the solvent which have been attributed to the formation of carbonic acid. It should be noted, however, that enzymes are not soluble in SCFs, therefore enzymatic catalysis in SCFs will always be heterogeneous.

Enzymes are proteins designed to fit a specific substrate(s). They have an active site which is tailor-made for the substrate. In a dense gas the enzyme molecule is becoming more rigid, which may be an advantage in the case of protein deactivation, namely the enzyme molecule is not prone to denaturation so quickly. At high pressure, spatial structure of many proteins may be significantly altered and they are denaturated with a loss in activity.

In SCFs there are direct effects of pressure on enzyme activity, which may lead to denaturation, and indirect effects of pressure on enzymatic activity. In the case of $SCCO_2$ only small direct effects of pressure with regard to enzyme inactivation are expected. Protein structure should retain on the whole and only local changes may occur. Those local changes may lead to another active state of a protein which may possess an altered activity, specificity, and stability. Pressure is also likely to affect the reaction performance indirectly by changing either the rate constant or the

reactants solubility. At higher pressures more solute-solvent interactions take place, resulting in a better solvent capacity.

Enzyme stability and activity in dense gases depend on: the source of the enzyme, the SCF, the water content of the enzyme/support/reaction mixture and the pressure and temperature of the reaction system. Therefore no theoretical prediction whether an enzyme should be active in a SCF or not can be made. Experimental study of the system behavior is necessary.

For the use of enzymes as biocatalysts in SCFs, the advantage that they are not bound to their natural role (they exhibit a high substrate tolerance and are not specifically required to work in water) is very important. The advantage that they act under mild conditions (pH about 5–8, typically around 7 and in a temperature range of 20–40 °C) may be sometimes turned into a drawback.

Enzymes as biocatalysts require narrow operation parameters. Elevated temperatures, as well as extreme pH, may lead to deactivation of the protein.

Many enzymatic reactions are prone to substrate or product inhibition and therefore must be carried out at lower substrate or product concentrations, a factor which limits the efficiency of the process. Whereas substrate inhibition can be circumvented easily by keeping the substrate concentration at low level through continuous addition, product inhibition is a more complicated problem. The gradual removal of product by physical means is usually difficult, but can be done elegantly with the use of dense gases as reaction media in a continuous process.

Enzymes in SCFs could either be used in their native form (powder, liquid . . .) or immobilized on a carrier (resin, sol–gel matrix . . .). The stability of an enzyme is dependent on its shape. It is also very important whether physical or chemical immobilization methods are applied.

The most of the research published to date deals with two problems: (1) conformation and stability in the SC environment (mainly CO_2) and the effect of pressure on reaction rate; (2) the effect of water/moisture content on the activity of enzyme.

6.3 Parameters Affecting Enzymatic Catalysis in Supercritical Fluids

6.3.1 Effect of Pressure

Aaltonen (1999) reported that apart from the direct conformational changes in enzymes, which may occur at very high pressures, pressure affects enzymatic reaction rates in SCFs in two ways. First, the reaction rate constant changes with pressure according to transition stage theory and standard thermodynamics. Theoretically, one can predict the effect of pressure on reaction rate if the reaction mechanism, the activation volumes and the compressibility factors are known. Second, the reaction rates may change with the density of SCFs because physical

parameters, as the dielectric constant, change with density. These changes may indirectly influence enzyme activity.

Certain enzymes show considerable apparent pressure activation (Dufour et al. 1995), while the majority of enzymes show deactivation with increasing pressure. Pressure can modify the catalytic behavior of enzyme by changing, for example, the rate-limiting step (Gross et al. 1993) or modulating the selectivity of the enzyme (Okamoto et al. 1991). Pressure affects the reaction rate by changing either the reactants solubility or the rate constant directly. This is of great advantage because it means that the solvent-power of the SCF can be adjusted for reaction performance. On the one hand, the solubility of substances increases with higher pressures because of a higher fluid-density and this is essential to bring the initial products in the reactor and remove the end-products from the reactor. On the other hand higher pressure normally results in higher reaction rates. Therefore a pressure increase is, in most cases, positive for enzymatic reactions, but for several enzymes deactivation occurs due to conformational changes in enzymes.

The influence of system's pressure on the stability of enzymes is not so significant within the pressure ranges of up to 30 MPa. Pressure-induced deactivation of enzymes takes place mostly at pressure exceeding 150 MPa. Reversible pressure denaturation mostly occurs at pressures below 300 MPa and higher pressure needed to cause irreversible denaturation (Cheftel 1991).

One of the advantages of using SCFs as enzymatic reaction media is separation of products from the reaction mixture with changing the pressure of the SCF. With the respect to the facts mentioned previously the solvent power of the SCF can be adjusted for running reactions. Products can be easily removed from the reactor. The effect of pressure on the reaction rate constant has not yet been determined but the effect on the overall production rate has been examined in several papers. Erickson et al. (1990) carried out transesterification of triglycerides, using lipase from *Rhizopus arrhizius*. The reactants used were trilaurin and palmitic acid and the pressure ranged from (10 to 30) MPa. A strong negative effect of pressure increase on the rate of palmitic acid incorporation into triglyceride was detected, especially in the near-critical region. The interesterification of trilaurin and myristic acid, catalysed by lipase, was investigated by Miller et al. (1991) in the pressure range (6–11) MPa. The interesterification rate and the overall rate (based on total trilaurin conversion) increased with increase in pressure; however, the interesterification rate increased much more rapidly than the overall rate, indicating that the selectivity of the reaction for interesterification over hydrolysis improved at higher pressures. The operational stability of enzymes in $SCCO_2$ is of crucial importance from the point of view of application. Miller et al. (1991) measured the interesterification rate over 80 h of continuous operation and observed no loss of activity of lipase. Cholesterol oxidase is stable at 10 MPa and 35 °C for at least 50 h (Randolph et al. 1988a, b). Pressure has also been found to have little effect on the stability of lipase from *Mucor miehei* in the range (13–18) MPa, causing only 10 % loss of activity (Marty et al. 1990) after 6 days at 40 °C, unlike temperature, effect which contributed to a 20 % loss at 60 °C. Additionally, in some cases a negative effect of pressure on the catalytic activity on biocatalysts in compressed gases may be

observed. The catalytic efficiency of subtilisin Carlsberg suspended in compressed propane, near-critical ethane, near-critical CO_2 and tert-amyl alcohol at constant temperature and pressure up to 30 MPa and fixed enzyme hydration was lowered (Fontes et al. 1998). In near-critical fluids an increase in pressure of only 20 MPa caused a sixfold decrease in the catalytic efficiency of subtilisin in CO_2.

In $SCCO_2$ the formation of carbamates is essential for lower enzymatic activity in this medium. Carbamates are the product of the reaction between basic free amino groups in enzymes and acidic $SCCO_2$ (Kamat et al. 1995b). On the other hand, lysozyme lipase unfolded and partially oligomerized in moist $SCCO_2$ at 80 °C and its denaturation was not caused by interaction with $SCCO_2$ but by heating the protein in the presence of water, as found by Weder (1984). When the lipid-coated lipase was employed in supercritical CHF_3 the enzyme activity (Mori et al. 2001) could switch on and off by adjusting pressure or temperature of the SC CHF_3.

The effect of pressure on the extent of conversion and the product composition in the enzyme-catalyzed hydrolysis of canola oil in $SCCO_2$ was investigated using lipase from *Mucor miehei* immobilized on macroporous anionic resin (Rezaei and Temelli 2001). A conversion of (63 – 67) % was obtained at (24–38) MPa. Monoglyceride production was favoured at 24 MPa. The amount of product obtained was higher at (24–38) MPa due to enhanced solubility of $SCCO_2$. The impact of operating conditions in the enzymatic esterification of *n*-octyl oleate catalysed by immobilized lipase from *Rhizomucor miehei*, was investigated (Laudani et al. 2005). The experimental evidence was that changing the pressure actually changed enzymatic reaction rate at constant substrate concentrations. The series of tests at various pressures were performed, in a constant volume reactor, keeping the substrate concentration constant. With the pressure increase the reaction rate decreased. The reason of this particular behavior could be explained, taking into account that increasing the pressure, at constant volume, the molar fraction of substrates decreases, reducing the initial reaction rate.

Moreover, investigations of *Mucor miehei* (Rantakyla and Aaltonen 1994) lipase-catalysed esterifications in $SCCO_2$ showed that a pressure increase from (10 to 25) MPa reduced the initial reaction rates, paralleling the decreasing mole fraction of substrates. It seems plausible that at higher pressure a more considerable water amount was extracted from the enzyme beads, which resulted in lower reaction yields, in according with the investigation made by Knez et al. (1995).

6.3.2 *Effect of Temperature*

According to numerous studies (Lin et al. 2006; Vermue et al. 1992; Varma and Madras 2007; Šabeder et al. 2005; Romero et al. 2005; Nagesha et al. 2004; Kumar et al. 2004; Fontes et al. 1998; Peres et al. 2003; Almeida et al. 1998; Overmeyer et al. 1999), temperature is probably the most important reaction parameter since it influences enzyme activity much more than pressure. Two effects are joined during

an increase in reaction temperature: The reaction rate increases with higher temperature, and enzyme activation/deactivation occurs. On pressure/temperature combination solubility of substrates and products are dependent and higher solubilities of substances in SCFs are achieved with increase in temperature. On the other hand, enzyme deactivation may occur at too high temperature. For these reasons optimal temperature for the enzyme activity and the one for the reaction performance are not necessarily the same. At the moment no correlation for the stability of the different types of enzymes with the temperature is available.

The thermal deactivation is also connected with changes in the water distribution in the system. The better stability of the lipase in the low-water-content environment is a consequence of the well-known fact that many reactions, which are responsible for the denaturation of enzymes, are hydrolytic reactions, and therefore, require water (Mattiasson and Aldercreutz 1991).

An increase in temperature at constant pressure may activate the enzyme. This activation may has solely a temperature effect or could be combined with one mediated by changes in solvation (Almeida et al. 1998). Thermal activation and deactivation (energy of activation and deactivation enthalpy, respectively) could be determined from the Arrhenius diagram. The ratio between the mass of inactive and active forms of the enzyme at the temperature at which the initial reaction rate was greatest is expressed via the deactivation constant. If energy of activation and deactivation enthalpy values is high this indicates that enzyme activity is influenced considerably by temperature.

Lipase from *Aspergillus niger* was incubated in $SCCO_2$ at 30 MPa and different temperatures (Knez et al. 2003). Its residual activity was optimal at 323 K. At higher temperatures a rapid decrease in activity was observed. This thermal deactivation was connected to changes in the water distribution in the system.

In microaqueous media, including SCFs, thermal stability of biocatalysts could also be improved. Reaction rate for subtilisin protease-catalyzed reactions increased up to 80 % in $SCCO_2$ (Pasta et al. 1989). Optimal temperature for esterification between *n*-butyric acid and isoamyl alcohol, catalyzed by porcine pancreas lipase, moved from 313 K at atmospheric pressure to 323 K in near-critical propane (Habulin and Knez 2001b). The effect of temperature was also investigated at various working pressures. The activity of *Rhizomucor miehei* lipase exhibits an optimum operating temperature for *n*-octyl oleate synthesis in the (70–80) °C range (Laudani et al. 2005).

6.3.3 *Effect of Water Activity*

Water concentration in the reaction system is one of the most important factors that influence activity of an enzyme, because they require a specific amount of water bound to them. Therefore, water is crucial for enzymes and affects enzyme action in various ways: by influencing enzyme structure via non-covalent binding and

disruption of hydrogen bonds; by facilitating reagent diffusion; and by influencing the reaction equilibrium (Krishna 2002).

When studying biocatalysts in non aqueous media, it is useful to be able to convert between water concentration and thermodynamic water activity (α_w). A set of relationships between water concentration and α_w for a range of polar solvents used in biocatalysis was presented by Bell et al. (1997). Their use is illustrated by the conversion of enzyme activity data from a water concentration to a α_w basis. This shows that α_w does not always predict the critical water level for high V_m values in polar solvents.

The ionization state of the enzyme and the α_w of the system are two important factors which affect catalytic activity in low-water organic solvents. Harper et al. (2001) studied the interaction between effects of α_w and the ionisation state of subtilisin Carlsberg, controlled by an organo-soluble dendrimer buffer. It was shown that α_w does not have a major effect on the relationship between rate of transesterification in toluene and buffer ratio, [sodium salt]/[acid]. The weak dependence on α_w (between 0.54 and 0.85) probably reflects compensating effects on ionisation equilibria for the buffer and the carboxyl groups on the enzyme.

$SCCO_2$ may dissolve (0.3–0.5) % (w/w) water, depending on the temperature and pressure, therefore use of an enzyme in pure $SCCO_2$ may lead to removal of the water, which is included or bonded to the enzyme. The quantity of the water removed is temperature and pressure depended and if it is too high this may lead to enzyme denaturation and loss of enzyme activity (Rezaei et al. 2007; Dijkstra et al. 2007; Peres et al. 2003, 2005; Fontes et al. 2002; Almeida et al. 1998).

If water acts as a substrate in an enzymatic reaction, optimal parameters for continuous reaction require, among others, enough moisture to compensate for complete reaction and sufficient enzyme moisture for losses due to water solubility in $SCCO_2$ (Hampson and Foglia 1999). However, if the water concentration in the supercritical medium is too high or if water is a product in the reaction the increased humidity may cause enzyme deactivation.

To the same extent salt hydrate pairs can control water activity and have a beneficial effect on both initial rates and conversion (Peres et al. 2003). Addition of organic and inorganic buffers to the microemulsions can retain the pH value of the system, and so stabilize the enzyme.

The subtilisin Carlsberg catalyzed transesterification of *N*-acetyl phenylalanine methyl ester (Aaltonen 1999), *N*-acetyl phenylalanine ethyl ester (Turner et al. 2001a, b), *N*-trifluoroacetyl phenylalanine methyl ester (Rezaei and Temelli 2001) and *N*-trifluoroacetyl phenylalanine ethyl ester (Schmitt-Rozieres et al. 2000) was studied in $SCCO_2$. The water content of the reaction affected the reactivity of the system; for the transesterification of the methyl esters with ethanol the optimum concentration of water was determined to be about 0.74 M, while during the transesterification of the ethyl esters with methanol it was about 1.3 M (Smallridge et al. 2002). Use of an enzyme in pure $SCCO_2$ may lead to removal of the water, which is included or bonded to the enzyme. The solubility of water in $SCCO_2$ can be calculated by Chrastil equation (Chrastil 1982):

$$c = \rho^k \cdot \exp\left(\frac{a}{T} + b\right) \tag{6.1}$$

where c is the solubility (g/L), ρ is the CO_2 density (g/L) and T the temperature (K). The calculated water parameters are k = 1.549, a = − 2,826.4 and b = − 0.807.

Some amount of water is necessary in the SCF because water-saturated CO_2 causes the inhibition of enzymes and consequent loss of activity. The optimal water concentration has to be determined for each enzyme separately. Enzymes require some specific amount of water to maintain their active conformation. Enzyme stability generally decreases with increasing water concentration, whereas their activities require some water to be present. Therefore, the water content has to be optimized in order to find the best balance between enzyme life and activity. Not surprisingly, all studies published so far pointed out the strong influence of moisture on enzymatic activity and reaction rates. Optimum water content in the support was estimated at 10 wt%, irrespective of the operating conditions (Marty et al. 1992), but this value may be contested in view of other reports (Leitgeb and Knez 1990). To prevent dehydration of the enzyme, the fluid in contact with the protein must contain water. The most hydrophilic hydrocarbons (e.g. hexane) dissolve 0.01 % water but $SCCO_2$ may dissolve as much as (0.3–0.35) % water. However, it is not the solubility of water itself but the partition of water between enzymatic support of an immobilized enzyme and the solvent ($SCCO_2$), which matters. Marty et al. (1992) carried out an extensive analysis of the partition of water between the enzymatic support of the immobilized enzyme and $SCCO_2$ as a function of pressure and temperature. They found that increasing temperature had a negative effect on the adsorption of water to the support but increasing pressure also had a similar effect. This is opposite of the results obtained by gas adsorption, which suggests that the solvation effect predominates over the vapor pressure effect. The same authors also extensively studied the influence of ethanol (entrainer) content in $SCCO_2$ and found that ethanol has a strong 'drying' effect on the enzyme support of the immobilized enzyme: indeed, the more hydrophilic the fluid, the more pronounced is the dehydration of the enzymes. Increasing water content, above the optimum level, adversely affects the overall performance. This appears to be related to hydrophilic hindrance of the hydrophobic substrate on its way to the active sites on the enzyme, and eventually makes the thermodynamic equilibrium less favorable (Basheer et al. 1995). Chulalaksananukul et al. (1993) measured the residual activity of lipase from *Mucor miehei* after a day in $SCCO_2$ at temperature range of (40–100) °C at various water concentrations. As the temperature rises, the enzyme molecule at first unfolds reversibly and then undergoes one or more reactions as following: formation of incorrect or scrambled structures, cleavage of disulfide bonds, deamination of trypsine residues, and hydrolysis of peptide bonds. Each process requires water and is therefore accelerated with increasing water concentration. Proteinase from *Carica papaya* latex was incubated in $SCCO_2$ for 24 h at 30 MPa and different temperatures (Knez et al. 2003). At high temperatures water was "extracted" from the enzyme microenvironment by the $SCCO_2$ and caused lower enzyme activity (one of the reasons for this effect). The crude proteinase

contained 1.53 % (w/w) water, while the proteinase incubated in $SCCO_2$ at 333 K contained only 0.99 % (w/w) water. Porcine pancreas lipase was used as biocatalyst for esterification (Habulin and Knez 2001b) between *n*-butyric acid and isoamyl alcohol in near critical propane at 313 K and 10 MPa. The same lipase preparation was used in the batch reactor for 10 reaction cycles. Conversion decreased to about half of the initial value after 10 reaction cycles. In the first two reaction cycles conversion was 34 % and after the 10th reaction cycle it was 17 %. In this case water, as a reaction product, remained with the enzyme in the reactor and its concentration increased with each reaction cycle. The reason for the decreased conversion was a phase split (two phases appeared). The apparent reaction rates decreased also as the reaction equilibrium shifted towards acids and alcohol formation. In contrast, the equilibrium was shifted towards ester formation in microaqueous media. It was confirmed that enzymes are active in the two-phase supercritical region as well (Knez et al. 2005). This depends on the type of reaction, which the enzyme catalyzes. Hydrolysis of sunflower oil (Primožič et al. 2003) in aqueous $SCCO_2$ was performed by the lipase from *Aspergillus niger* at 323 K and 20 MPa. A conversion obtained at optimal reaction conditions was about 86 %.

The presence of optimal levels of water in enzymatic hydrolysis reactions in a $SCCO_2$ medium is critical not only as a substrate but also in order to maintain the catalytic activity of the enzyme (Martinez et al. 2002). When an enzyme is used in $SCCO_2$ in a batch system, with each expansion, a certain amount of water is removed from the enzyme preparation. To avoid enzyme deactivation as a consequence, water should be added to the system at the start of the reaction. The effect of the water on enzyme activity of immobilized lipase from *C. antarctica* (Novozym 435) in subcritical 1,1,1,2-tetrafluoroethane (R134a) was investigated varying the amount of water added between (7 and 56) μL, at 40 °C, 4 MPa. A decrease in conversion with increasing water content was observed. The conversion with 7 μL of water added was 73 % (5 h) and decreased to 66 % with 56 μL of water added. Enzyme needs a small amount of water to retain its active three-dimensional conformational state, even when the enzyme is covalently bound to a support. Since water is a product of the reaction, excess water would reverse the reaction and reduce the conversion of the acid (Gang et al. 2007).

6.3.4 Effects of Pressurization and Depressurization

The influence of pressurization-depressurization steps in batch reactors on the enzyme activity is of importance to many researchers (Knez et al. 2001). Pressurizing an enzyme usually does not play an important role. Depressurization is usually the step, which influences residual enzyme activity. One must take into account that at too fast expansion, unfolding of the enzyme may destroy its structure. When the enzyme is pressurized in the SCF, the SCF permeates through the enzymes by diffusion, which is a relatively slow process. After a certain time, the enzymes are saturated with the SCFs. With slow depressurization the fluid has

enough time to leave the enzyme and the bulk. On the other hand, with too fast expansion the fluid cannot leave the enzyme fast enough, which causes a higher fluid pressure in the enzyme in the comparison to the bulk (Knez et al. 2001).

For practical use of enzymes in batch systems using SCFs as reaction media, slow expansion, following by the temperature decrease is recommended.

Transition from supercritical to normal condition is "enzyme friendly", because of the continuous density change. On the other hand, entering the two-phase region and expanding the liquid part of the fluid causes evaporation of the fluid, which is accompanied by a large change in density. This volume-expansion causes unfolding of the enzyme.

Exposure of enzyme preparation to liquid propane increases the enzyme activity compared to the non-solvent system by four- to nine-times dependent on the used lipase (Habulin and Knez 2001b).

Depressurization is of importance when using the benefit of SCFs for simple downstream processing. In this case by operating a cascade of depressurizations (with a possible change in temperature) product fractionation can be achieved (Romero et al. 2005). The activity of cross-linked enzyme aggregate of *C. antarctica* lipase B after one and four pressurization/depressurization steps was checked. No significant differences between the results for the conversion before and after pressurization/depressurization steps were observed (Dijkstra et al. 2007). Similar results were obtained for cross-linked enzyme crystals of *C. antarctica* lipase B (ChiroCLEC TM -CAB) where the decrease in activity from 96 % conversion in the first cycle to only 50 % conversion in third cycle was observed (Dijkstra et al. 2007).

Oliveira et al. (2006) studied the effect of compression/expansion cycles on enzyme activity in all solvents. Influence of depressurization rate on the activity of Lipozyme IM (immobilized lipase from *M. miehei*) and Novozym 435 (lipase from *C. antarctica*) exposed to compressed CO_2, propane and *n*-butane were investigated. Treatment of Lipozyme IM in CO_2 led to the highest activity losses (up to 14 %), followed by propane (maximum activity loss 8.9 %) and in a much lesser extent by *n*-butane (maximum 3.6 %). For Novozym 435 CO_2 also had a deleterious effect on its activity, though in a lesser degree compared to Lipozyme IM. Treatment of Novozym 435 in compressed propane and n-butane improved enzyme activity for all experimental conditions, with resulting activity gains as high as (14 and 22) %, respectively. Thermogravimetric analyses show that the thermal profiles of Novozym 435 treated in *n*-butane and in CO_2 are similar to the untreated enzyme. They found out that the magnitude of pressure (or reduced density), temperature, decompression rate and exposure time needed to affect the lipase activity strongly depends on the nature and the source of enzyme and, mainly, whether the enzyme is in its native or immobilized form.

Since the immobilization of enzyme prevents conformational changes that could occur due to chemical interaction of SCFs with the enzyme and consequently improves its stability, it would be expected that native enzyme are more susceptible to influence of depressurization/pressurization step on activity. But it depends also on enzyme species and the reaction media.

6.4 Enzyme Stability in SCFs

Enzymes are proteins designed to fit a specific substrate(s). They have the active site which is tailor-made for the substrate. Generally, enzymes are classified into 6 major classes, based on the nature of the catalyzed reaction. Elevated temperatures, as well as extreme pH, may lead to deactivation of the protein (Habulin et al. 2007).

It is very difficult to predict the stability and activity of an enzyme in any SCF. Beside the influence of the medium there are numerous other parameters that influence the stability of the enzymes in SCFs such as water activity, pressure, temperature, pH, depressurization, etc.

Extensive research has been done in this field (Nakamura et al. 1986; Kamat et al. 1995a; Fontes et al. 1998) but the results obtained are very difficult to compare because of the non-standard methods used by the investigators and the different aims of the studies performed. Usually, improved enzyme activity was desired, namely, enzyme activity in $SCCO_2$ was enhanced via changes in acid–base conditions by using ion-exchange materials (solid H^+/Na^+ buffer pairs in zeolite). The latter were selected on the basis of the response of an organosoluble acid–base indicator (Harper and Barreiros 2002). In other cases, enzyme inactivation was the goal of the study, e.g. enzyme inactivation in a buffer system with microbubbles of $SCCO_2$ (Yoshimura et al. 2002).

Catalytic activity of enzyme or enzyme preparation could be reduced or destroyed due to formation of carbamates between CO_2 and lysine residues on the enzyme surface and also, because of the presence of water in the reaction system or enzyme/enzyme preparation, due to formation of carbonic acid (Hobbs and Thomas 2007). The use of water/$SCCO_2$ medium could lead to protein denaturation and/or its structural modification because the solubilisation of CO_2 caused intense acidification of the medium and consecutively a change in pH. Protein degradation depends a great deal on its physico-chemical structures (Vezzù et al. 2008).

Substrates, as well as end-products, may cause enzyme inhibition if they block the active center of the enzyme. In such cases no activity and selectivity of the enzyme is available and reaction conditions must be improved, for example by optimizing the amount of substrates and end-products.

Early investigations (Randolph et al. 1985; Hammond et al. 1985; Nakamura et al. 1986) demonstrated that certain enzymes are active in $SCCO_2$. Randolph et al. (1988a, 1988b) first studied the conformation of several spin-labelled variants of cholesterol oxidase in $SCCO_2$ and concluded that these proteins were not influenced by the $SCCO_2$ environment; a similar result has recently been obtained for lipase (Miller et al. 1991). In contrast, Kasche et al. (1988) reported that alpha-chymotrypsin, trypsin and penicillin amidase were partially denaturated by $SCCO_2$ and suggested that it might be a result of the decompression process. Still, no *in situ* measurements were conducted to substantiate this suggestion.

Zagrobelny and Bright (1992) carried out a more detailed examination of the same problem. The conformation of trypsin in $SCCO_2$ was studied at the pressure

range of (5–25) MPa and the conformational changes of trypsin *in situ*, as a function of pressure, was monitored. Their results clearly demonstrate that: (1) significant changes in protein conformation can be induced by SC solvents; (2) most of the conformational changes occur during compression; (3) the native trypsin conformation is only slightly more stable than the unfolded form.

Performance characteristics, specific rates of conversion and yield factors are essential for rating any technological process. On the whole, enzymatic reactions in $SCCO_2$ proceed at rates similar to those of organic solvents such as *n*-hexane (Marty et al. 1990, 1992) and cyclohexane (Miller et al. 1991) and similar rates of processing and enzyme stability are ensured. Supercritical technologies offer important advantages—such as ecological friendliness and product fractionation - over organic solvent ones. The benefits can also be linked with the direct micronisation and crystallization caused by the $SCCO_2$ fluid expansion. In addition, CO_2 does not usually oxidize substrates and products, allowing thus the process to be operated at a moderate temperature of only 40 °C.

Many enzymes are stable in SCFs; still one should pay considerable attention to finding the correct reaction conditions for each substrate/enzyme/SCF system. For example, although successful reactions have been reported with Subtilisin Carlsberg protease and *Candida* lipases in $SCCO_2$, there is also evidence for their instability (Kamat et al. 1992, 1995b; De Carvalho et al. 1994) or the existence of a narrow pressure range of activity (Ikushima et al. 1995, 1996). These enzymes are fairly stable in other SCFs such as fluoroform, ethylene, ethane, propane, and sulphur hexafluoride (Kamat et al. 1992). Immobilized *Mucor miehei* lipase appears to be very stable in $SCCO_2$. It is a monomeric enzyme with three stabilizing disulfide bonds (Jensen et al. 1987), which may play a role in maintaining its activity in $SCCO_2$.

Enzymes, as biocatalysts, should retain their activity for a considerable period of time. For example, the activity of the lipase from *Candida antarctica* for the production of isoamyl acetate in $SCCO_2$ was studied in a tubular reactor by measuring the esterification extent at the stationary state (Romero et al. 2005). The yield of isoamyl acetate was 100 % for 30 days, and then slowly decreased. Habulin et al. (1996b) found similar results with immobilized *Rhizomucor miehie* lipase, reporting a 4 % decrease in conversion after 1 month of treatment. Cholesterol oxidase from different sources can exhibit different stabilities in $SCCO_2$ (Randolph et al. 1988a, b). By cholesterol oxidation, cholesterol oxidase from *Gloecysticum* retained its activity for 3 days and the one from *Streptomyces sp.* for only 1 h. In some cases half-life of the biocatalysts under pressure could be increased, as observed by Lozano et al. (1996). The half-life of alpha-chymotrypsin increased with the pressure increase from 8 to 15 MPa.

Residual activity of immobilized lipase from *C. antarctica* was studied also in other SCFs. Gang and coworkers (2007) found out that subcritical R134a treatment led to a significant increase of Novozym 435 activity and a maximum residual activity of 300 % was measured at 4 MPa, 30 °C after 7 h incubation. No deactivation of Novozym 435 treated with subcritical R134a under different operation factors (pressure 2–8 MPa, temperature 30–60 °C, incubation time 1–12 h,

water content 1:1, 1:2, 1:5 enzyme/water, depressurization rate 4 MPa/1 min, 4 MPa/30 min, 4 MPa/90 min) was observed.

6.4.1 Non-immobilized Enzymes

Non-immobilized lipases from different sources were first incubated, at defined conditions, in $SCCO_2$ and in sub-critical propane, and afterwards used as biocatalysts. A comparison with the reaction, catalyzed by non-incubated (fresh) enzyme showed that these lipases were stable in the two media examined (Habulin and Knez 2001b).

Lipases, in their non-immobilized form, from the same sources as in the previous case were used as biocatalysts for the butyric acid and ethanol esterification in CO_2 and propane at high pressure. Initial reaction rates strongly depend upon the choice of the solvent used for the reaction performance. A change in the enzyme activity was not the only factor leading to differences in the initial reaction rates. Other effects, such as water partition between the enzyme and the reaction mixture, solvent power of the reaction medium or dielectric constant affected rate of reaction, too. Activity of biocatalysts is strongly dependent on the dielectric constant of the solvent and it was reported that a major change in protein flexibility occurs when solvent dielectric constant increases from 1 to 10 (Kamat et al. 1992; Affleck et al. 1992). It has been suggested that dielectric constant can be used to predict the specificity of an enzyme-catalyzed reaction. Russell and Beckman (1991) showed that in a Subtilisin carlsberg-catalyzed, or *Aspergillus* protease-catalyzed, transesterification reaction between *N*-acetyl- (L or D)-phenylalanine ethyl ester and methanol pressure-induced changes in the dielectric constant of fluoroform gave rise to predictable changes in the enantioselectivity of both enzymes.

Activity of in $SCCO_2$ preincubated non-immobilized proteinase from *Carica papaya* latex (at 30 MPa) changed in comparison to the activity of the crude enzyme preparation (Habulin et al. 2005a, b). These changes were connected with water distribution in the system. The measurements were performed by the Karl-Fisher method and it was demonstrated that the crude proteinase contained 1.53 % of water, while the in $SCCO_2$ incubated proteinase contained only 0.99 % of water. Water plays a vital role in the non-covalent interactions that allow the enzyme to retain its native conformation. In the complete absence of water, enzymes cannot maintain an active conformation, thus hindering their ability to function as catalysts (Mesiano et al. 1999). In $SCCO_2$, at temperatures above 40 °C, a proteinase activity decrease appeared, while at atmospheric pressure better temperature stability of the biocatalyst was observed.

The residual activity of proteinase, which was incubated in near-critical propane and dimethyl ether (DME) at 30 MPa, was lower than the activity of the crude enzyme preparation. In DME the original value was reached with the thermal

activation at (50 and 60) °C. It is evident that in this case physical properties of SCFs have a dramatic effect on enzyme stability.

6.4.2 Immobilized Enzymes

Immobilization is also important to prevent conformational changes that could occur due to chemical interaction of SCFs with the enzyme (Kamat et al. 1995b) as well as during pressurization or depressurization.

Quite often when an enzyme is immobilized its operational stability is improved. Immobilized enzymes are expected to be more stable in sub- and supercritical media and therefore their activity should be almost unchanged.

Immobilized lipase from *Rhizomucor miehei* was first incubated in different gases ($SCCO_2$, sub-critical butane and mixture of *n*-propane/*n*-butane) at 35 °C and 10 MPa and afterwards used as a biocatalyst. No difference in the residual activity was observed (Krmelj et al. 1999). When the same lipase was used as biocatalyst in the same dense gases as before but at 50 °C and pressure from (10 to 30) MPa, the highest reaction rates were achieved in $SCCO_2$. In *n*-butane and in the mixture of *n*-propane/*n*-butane reaction rates were almost the same. At given temperature and pressure *n*-butane and the mixture of *n*-propane/*n*-butane are at sub-critical conditions, while CO_2 exists at supercritical state. Reaction rates in *n*-butane and in the mixture of *n*-propane/*n*-butane did not change with the pressure rise from (10 till 30) MPa while in the same pressure range initial reaction rates in $SCCO_2$ increased with higher pressure for about 70 %.

The immobilization of cellulase in silica aerogel matrix has shown to be very efficient as it has improved its biocatalytic properties, such as activity, stability, as well as its repeated application for hydrolysis of carboxymethyl cellulose (Paljevac et al. 2007). The activity of the immobilized cellulase rose for about 110 % in comparison to the activity of native cellulase in the reaction performed at atmospheric pressure. Incubation of the immobilized enzyme in $SCCO_2$ (at 10 MPa and 35 °C), prior the hydrolytic reaction, improved residual activity up to 461 %. The thermal stability of immobilized cellulase on aerogel matrix enzyme was improved since residual activity after incubation at 110 °C in $SCCO_2$ was still higher than 150 %.

6.4.3 Reactions Using Whole Cells

Compared with isolated enzymes, whole cell catalysts can be much more readily and inexpensively prepared. There is no need for downstream processing and purification of enzymes. Generally, they are more stable in the long-term than free enzymes, because enzymes in cells are protected from the external environment. Rapid advance in life science (recombinant DNA technique) have greatly

increased the availability of whole cell biocatalysts. Nonaqueous whole cell-based biocatalysis, as an important part of biocatalysis, which can enhance the solubility of poorly soluble compounds, change the thermodynamic equilibrium in favor of product synthesis, etc.

For example, the resting cells of a *Geotrichum candidum* fungus were used for the asymmetric reduction of various ketones in $SCCO_2$ (Matsuda et al. 2000; Nakamura and Matsuda 1998; Nakamura et al. 1999).

The cells of *Bacillus megaterium* were employed for the CO_2 fixation reaction (decarboxylase catalysed) from pyrrole to pyrrole-2-carboxylate in $SCCO_2$. As shown by Matsuda et al. (2001b) higher yields were obtained under supercritical conditions (55 %) than at atmospheric pressure (7 %).

There are not many studies done in which the whole cells were used as biocatalyst for the reaction in supercritical fluids. However, $SCCO_2$ can serve as a solvent for the extraction of intracellular components from microbial cells or for isolation of products from the reaction mixture in the production of biomass (Debs-Louka et al. 1999; Erkmen 2003; Hong and Pyun 2001).

Activity of cellulase and α-amylase from *Hortaea werneckii* after cell treatment with supercritical carbon dioxide was studied and it was shown that a significantly high amount of proteins was released from *H. werneckii* cells during the $SCCO_2$ treatment. The residual activity of both enzymes examined decreased with prolonged treatment time of *H. werneckii* cell suspension in $SCCO_2$, but both could be still used as biocatalysts in this medium. The reason for such activity loss could be due to longer exposure to high pressure which could cause deactivation of enzymes or most probably due to the pH change of cell suspension owing to formation of carbonic acid. Since both α-amylase and cellulase are multimeric enzymes, the inactivation of enzymes could also be due to subunit dissociation (Leitgeb et al. 2013).

6.5 High-Pressure Enzyme Reactors

Enzymatic reactions in sub- and supercritical fluids have been performed in different high-pressure reactors - batch-stirred-tank, extractive semi-batch-, recirculating batch-semicontinuous flow-, continuous packed-bed- and continuous-membrane - and immobilized and native enzymes were used as biocatalysts. The main advantage of immobilized enzyme usage in high pressure reactors is the possibility to re-use it for more reaction cycles.

For screening enzymatic reactions in dense gases, batch-stirred-tank reactors are preferentially used (Knez et al. 1995, 1998; Erickson et al. 1990; Knez and Habulin 1994, 2002; Habulin et al. 1996a). Applications of high-pressure membrane reactor with a flat-shape membrane and membrane reactor with tubular membrane were also reported (Habulin et al. 2005a; Gumi et al. 2007; Hernandez et al. 2006).

In order to shift reaction equilibrium toward product formation, extractive batch-reactors for continuous extraction of products from reaction mixtures were used (Gunnlaugsdottir and Sivik 1995).

Recirculating batch-reactors are mainly used for kinetic studies of enzymatic reactions in dense gases (Yu et al. 1992). The advantage of this type of reactor is that the pure gas can be compressed. The disadvantage of semicontinuous flow reactors is that the concentration of substrates in dense gases cannot be varied, and that with changes of pressure and temperature, precipitation of substrates or products in the reactor can occur (Miller et al. 1991; Bauza et al. 2002).

Rezaei and Temelli (2000) report on application of continuous high-pressure fixed-bed enzymatic reactor. Krmelj et al. (1999) performed lipase-catalyzed continuous synthesis of oleyl oleate at 313 K and 15 MPa in *n*-butane and CO_2 in a continuous packed bad reactor. Conversion in the system where *n*-butane was used as a reaction medium, decreased continuously while it was constant in the system where $SCCO_2$ was used. Other reactions were also performed in continuous high-pressure fixed-bed enzymatic reactor e.g. King et al. (2001), Rezaei and Temelli (2000).

Hydrolysis of blackcurrant oil, catalyzed by the immobilized lipase from *Mucor miehei* was performed in a continuous flow reactor at (10–28) MPa and (303–323) K (Sovová and Zarevućka 2003). Lipase stability was very good with no activity reduction observed during the long-time experiment. At optimal conditions, a complete hydrolysis of oil was achieved.

Kinetic studies of enzymatic reactions in dense gases may be performed in high-pressure recirculating batch reactors. In the high-pressure semicontinuous-flow reactors, the substrates saturated dense gas is fed continuously through the enzyme bed (Aaltonen 1999).

Each type of high-pressure reactors has its own advantages and disadvantages. For the high-pressure batch reactor the main disadvantage is the expansion-induced deactivation in the case of enzyme reuse. For the high-pressure semicontinuous flow reactors the main disadvantage is that the concentration of substrates in dense gases cannot be varied, and that with changes of pressure and temperature, precipitation of substrates or products in the reactor can occur. To avoid such disadvantages, a high-pressure continuous membrane reactor was advocated.

With respect to the low product specific catalyst costs, continuously operated biochemical systems are the most important from industrial point of view (Vasić-Rački et al. 1998). For operating in SC conditions, appropriate membranes have to be used. Immobilized *Candida antarctica* lipase B was used as catalyst to synthesize butyl butyrate from butyl vinyl ester and 1-butanol in $SCCO_2$. Active ceramic membranes were applied for continuous butyl butyrate synthesis (Lozano et al. 2004). The application of tubular ceramic membranes in high-pressure reaction systems was studied also in the case of carboxy methyl cellulose hydrolysis at atmospheric pressure and in a biphasic medium ($SCCO_2/H_2O$). The reaction was catalyzed with covalent linked cellulase from *Humicola insulens* on the surface of a ceramic membrane. The reaction carried out in the biphasic medium gave higher

productivity than the reaction, performed at atmospheric pressure (Primožič et al. 2009).

Conclusions

Studies on application of dense gases as solvents for chemical and biochemical reactions are at present an interesting research topic as can be observed from the yearly increase in publications since 1985. Still, application of dense gases as "green solvents" for biochemical reactions has not yet been realized on industrial scale. On the one hand, that could be a result of the instability and deactivation of enzymes under pressure, on the other hand—a result of a lack of fundamental knowledge and economic benefits.

The high solubility of several substrates, and the high activity of enzymes, in water, and taking into consideration that at present water is the cheapest solvent, can explain why there are no enzyme biotransformations performed in SCFs on industrial scale till now.

Due to high raw oil price and to the increase of organic solvent prices, the demand on use of new solvents increases. Therefore, research on the application f new reaction media like sub- and supercritical fluids, fluorinated solvents, ionic liquids, water and solvent free reaction systems is very important.

Production of single enantiomers instead of racemic mixtures is becoming more and more important in the pharmaceutical and agrochemical industry because, in most cases only one of the two enantiomers has the desired activity, whereas no activity or even undesirable side effects reside in the other enantiomer. The stereospecificity of an enzyme depends largely on the structure of the substrate, interaction at the active site and on the reaction conditions. Stereoselectivity of reaction depends on a number of factors such as differentiation of enantiotopes, differentiation of enantiomers, type of substrate, biochemical interaction of the substrates with the enzyme, steric interaction of the substrates, competition between two different substrates, nature and availability of the active site for stereoselective action, presence of water and nature of solvents based on polarity and supercritical state (Muralidhar et al. 2002). The resolution method mainly applied today is based on enzymatic conversion of one enantiomer of the racemate while the other remains unaltered due to a much slower conversion rate. Because enzymes have extremely high selectivity, and owing to the great importance of enantioselective synthesis or enantiomeric resolution in the pharmaceutical industry, a most intense research in this area can be expected, along with minimizing the use of substances and maximizing their effect.

Studies in the field of biochemical engineering should open a new research avenue on enzyme species, which could "survive" at unconventional conditions (high pressure and/or high temperature). Such enzymes can be isolated

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from extremophilles (deep see organisms and microorganisms, microorganisms from hot springs, high sulphur springs microorganisms, high salts environments, high pressure and low temperature environments microorganisms, etc.) that could lead to new products with special properties and new process developments.

Generally, the biochemistry at unconventional conditions offers research opportunities without borders. At the moment, there is practically no research on application of whole cells or cell debris as biocatalysts in sub- and in supercritical fluids.

Sub- and supercritical fluids (dense gases) can be accepted as green solvents for biochemical reactions, due to their availability in the environment and because there are no residues in the products. The processing of substances in membrane bioreactors offers the possibility of reaction and separation processes integration. In addition, use of dense gases gives the possibility to fractionate the products and to formulate the product in a single step. Lastly, because of intensive development of equipment producers, there is no limitation for scale up to industrial scale.

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Chapter 7
Advances in Analytical and Preparative Supercritical Fluid Chromatography. Food and Nutraceutical Applications

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7.1 Supercritical Fluid Chromatography Overview and Brief History

7.1.1 Introduction

Supercritical fluid chromatography (SFC) uses a solvent under subcritical/supercritical fluid conditions as the mobile phase. It is a burgeoning technology that combines some of the desirable features of gas (GC) and liquid chromatography (LC). Figure 7.1 shows a classification of chromatography based on Taylor (2009a).

SFC is a relatively recent chromatographic technique and there is significant research currently underway. SFC has been applied to a wide range of compounds, including natural products, drugs, foods, pesticides and herbicides, surfactants, polymers and polymer additives, fossil fuels, petroleum and explosives and propellants (Salvador 1996). SFC has some important advantages over GC and/or LC:

- SFC permits separation of compounds that are not conveniently handled using GC or LC. GC is unsuitable for non-volatile or thermally unstable compounds. LC cannot be easily employed for compounds with functional groups that cannot be detected by either spectroscopic or electrochemical detectors used in LC. Even though LC is suitable to analysis of thermally unstable or non-volatile compounds, it generates large quantities of waste organic solvent which needs to be managed. This issue, combined with more stringent environmental regulations has been a key aspect in the rise in popularity of SFC for some applications. As for Supercritical Fluid Extraction (SFE), SFC has gained a reputation as a green technique or environmentally friendly technology, when

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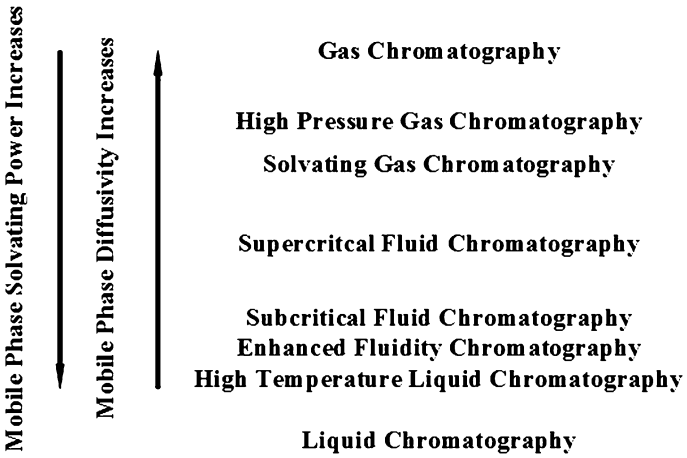

Fig. 7.1 Classification of Chromatography based on the work of Taylor (2009a)

the mobile phase used is carbon dioxide (CO_2). Although the CO_2 employed is a greenhouse gas, it is collected as a by-product of other chemical reactions and can be substantially recirculated within the process. Therefore, SFC doesn't contribute new CO_2 to the environment.

- SFC can separate some high molecular weight compounds, polymers and large biological molecules at reasonably low temperature (Hoffman and Taylor 2002; Jentoft and Gouw 1976; Kithinji et al. 1990; Lesellier 1999). However, it is best suited to the separation of non to low polarity, small to medium molecular weight compounds, and in particular lipids and lipophilic natural products and pharmaceuticals.
- SFC is a versatile technique because both GC and LC detectors are applicable. However, to choose the best detector for each application the nature of the sample, the mobile phase composition, the column type, and the flow rate must be taken into account. Mass spectrometers are also more easily adapted as a detector for SFC than for LC.
- SFC is inherently a higher throughput process than both GC and LC (2–5 times). SFC allows steep gradients, is tolerant of significant amounts of water, allows very long columns or series of columns or very small particles with modest pressure drops (Gere 1983).

As a conclusion, SFC will not replace LC or GC, but complement them particularly for the range of compounds that it is best suited to. Much of the research in the area during the last few years has been trying to adapt equipment and gain knowledge about SFC processes such as new columns, new detectors, better sample handling, fundamental studies of the mechanisms involved in SFC separations and applications. As stated by Taylor, "most of research in the field is of an 'evolutionary' rather than a 'revolutionary nature' (Taylor 2009a)".

SFC, and closely related methods, can be found in the literature under different names apart from supercritical fluid chromatography. Terms also used are Subcritical Fluid, Near-Critical Fluid, High-Temperature Liquid, Super-Heated Liquid, and Enhanced-Fluidity Liquid Chromatography. In early days of SFC, ultra high performance gas chromatography HPGC and Dense Gas Chromatography were used. As in supercritical fluid extraction, research done under subcritical conditions is included as SFC as conditions below but close to the critical point share many similar properties.

Evolution of SFC. The evolution of SFC can be divided into two different parts; early stages (1960s and 1970s), where a relatively small group of researchers employed this technology; and from 1979 to the current date, when commercial equipment became available and SFC development started.

In the early days, the presently accepted terminology "supercritical fluid chromatography (SFC)" was not established, and various terms were used such as (ultra) HPGC and dense gas chromatography (DGC). In 1958, Lovelock (Bertoncini et al. 2001), proposed the use of solvents in their critical state as the chromatographic mobile phases in order to extend the scope of gas chromatography to include ionic compounds which are otherwise non-volatile.

However, it was Klesper et al. (1962) who first carried out experiments with "dense gas" chromatography, as they named it. They used dichlorodifluoromethane and monochlorodifluoromethane under pressures between (70 and 100 atm) as mobile phases for the elution of nickel porphyrin isomers. They suggested that the increased mobility observed with increasing mobile phase pressure should permit the analysis of high molecular weight compounds at lower temperatures than GC. In the following years SFC started to be taken into account. The number of groups involved with this "new" chromatography was small but very active. Karayannis et al. showed how to control the column back pressure and the flow rate independently and described a UV detector with a cell operating under pressure (Karayannis et al. 1968). In 1966, Giddings (Giddings 1966; Giddings et al. 1966) developed a theoretical basis for the effect of pressure increase in gas chromatography. Later, Giddings et al. (1969) carried out chromatographic separations under very high pressure, up to 2,000 atm using helium, nitrogen, carbon dioxide, and ammonia as mobile phases to separate nucleosides, nucleotides, purines, proteins, peptides, amino acids, sugars, terpenes, and steroids.

Sie et al. (Sie and Rijnders 1967a, b, c) published a series of articles on HPGC in 1966 and 1967. They used supercritical CO_2 as the mobile phase and discussed fluid-solid and fluid-liquid separation modes. They developed a sophisticated pneumatically operated injector in order to inject a sample under high-pressure and high-temperature conditions. It is also unique that they used a UV absorption detector with a quartz cell that was equipped with a gas-liquid separator and detection was carried out under atmospheric pressure.

After the first years focused on mobile phase experimentation, research groups moved to develop their own equipment for ongoing research. In 1968, Klesper's group, reported a new SFC system (Karayannis et al. 1968). The system was equipped with a mechanical backpressure regulator that could control the column

outlet pressure independent of the flow rate. The detector was a filter photometer with a high-pressure flow cell. It should be noted that the detector used in HPLC at that time was a simple single-wavelength photometer with a low-pressure Hg discharge lamp as the light source which emits UV light at 254 nm (Snyder and Kirkland 1979). In the early 1970s, Jentoft and Gouw developed pressure-programmed SFC and applied it to separation of wide molecular weight range samples of polynuclear aromatic hydrocarbons and of styrene oligomers (Jentoft and Gouw 1972). They also designed and built an automatic fraction collector (Gouw and Jentoft 1972).

In the late 1970s, analytical and preparative SFC parted ways. All preparative applications of chromatography require that the columns can provide a significant production rate of the purified fractions, hence that they have a sufficiently large cross-section area. So, packed columns have always been used in preparative applications (Perrut 1994). Packing columns with finer particles certainly provides a higher separation efficiency than packing them with coarser ones but it was feared that it would give them too low permeability and, because CO_2 is compressible, that a significant pressure drop take place along the column, in spite of the low viscosity of CO_2. Accordingly, packed columns were abandoned in favour of 50 μm i.d. open tubular columns for analytical SFC, which had became successful and popular in gas chromatography.

"Widespread awareness of SFC", quoting Berger et al. (2010), occurred after Hewlett Packard (HP, now Agilent Technologies) presented a series of papers at the 1979 Pittsburgh Conference and introduced an SFC modification kit for the model 1,084 high performance liquid chromatography (HPLC) system, in 1981. This was a packed-column instrument with independent flow, composition, pressure, and temperature control. Detection was by UV, flame ionization detection (FID) and mass spectrometry (MS).

Preparative SFC began in the early 1980s with Perrut patenting preparative SFC with cyclone separators, for petroleum applications, using pure CO_2 as the mobile phase, in 1982 (Perrut 1984a). With the patents of Perrut (1984a, b), Perrut et al. (1982) a growing interest in SFC in the laboratory-, pilot- and industrial-scale could be observed. In 1992, Gilson and Hewlett-Packard both introduced commercial analytical-scale SFC systems. Their hardware used binary, reciprocating pumps, electronic back pressure regulators, UV detectors, and computer control of all variables. The HP system ran with both, packed and capillary columns.

Capillary or open-tubular SFC was commercialized around 1986 although they were known early (Novotny et al. 1981). Capillary columns dominated the development of both theory and instrumentation in SFC for the next 5–7 years. Capillary instruments were very similar to those used in GC, but used a syringe pump as a pressure source, and a fixed restrictor to limit flow through small-internal-diameter open-tubular columns. The majority of applications used pressure programming with pure CO_2 as the mobile phase. Detection was FID but many other detectors were used, including MS. Columns were mostly 50-μm i.d. fused silica. Most pioneers from the pharmaceutical industry who tested the available instrumentation in the 1980s found the technology was very limited, because of its poor

reproducibility and limited application range. Thus, in the early 1990s, SFC was closed to be forgotten because of lack of application to more polar analytes in the pharmaceutical market (Harris 2002; Phinney 2000). By 1997, it was clear that the future of SFC would be packed columns employed to separate moderately polar compounds with carbon dioxide as the mobile phase and using spectroscopic detectors. After that SFC has been carried out mainly employing packed columns, self-packed or available commercially.

Semipreparative SFC took off after Berger Instruments, headed by one of the modern developers of SFC, Dr. Terry Berger, introduced the semipreparative AutoPrep system for achiral library purification and the MultiGram II system for chiral-like stacked injections. These were basically scaled up versions of the analytical hardware, with a new kind of phase separator. Columns were 2–3 cm i.d., with packings identical to analytical columns.

As more companies began to manufacture equipment, SFC became more popular. Today, pharmaceutical applications continue to be most prevalent use of SFC (see Fig. 7.2). Food and natural products represent the next largest body of work. Major themes are the isolation and characterization of high value added foodstuff, fragrances, and flavour compound from novel natural materials or agricultural by-products. The areas of food, natural products, and pharmaceutical separation science converge in the area of so-called nutraceuticals. The final major category consists of environmental applications, both as an extraction technique for environmental analysis and as a possible remediation strategy for removing contaminants that otherwise would be too expensive to recover. Analytical applications dominate the use of SFC for now, with only a few known applications of industrial scale preparative chromatography (Pharma 2013). There is reluctance to use high pressure processes at industrial scale due to high initial capital costs and perceived safety considerations. Large industrial applications using supercritical fluids as extraction solvents are well established, operate economically, and have a good safety record, but large scale applications of SFC are still some steps behind.

Since the late 1990s, SFC research has been focused on expansion of application areas associated with development of column technology. In the twenty-first century, advances in column and mobile phase chemistry finally allowed the analysis of biomolecules that were previously difficult to separate by SFC, such as hydrophobic metabolites (Matsubara et al. 2010).

Publications describing advances in SFC have decreased in number compared to previous periods. This is consistent with the move of SFC from academic and development laboratories into industrial applications particularly in the pharmaceutical industry. The majority of publications describing detection revolve around "informative" spectroscopic and spectrometric detectors, which provide structural information about eluted analytes (See Fig. 7.3). The Green Chemistry Group (Oakmont, USA) has been promoting the International Conference on Packed Column SFC annually since 2008, specifically focused on SFC, because of its low visibility in general supercritical fluid conferences.

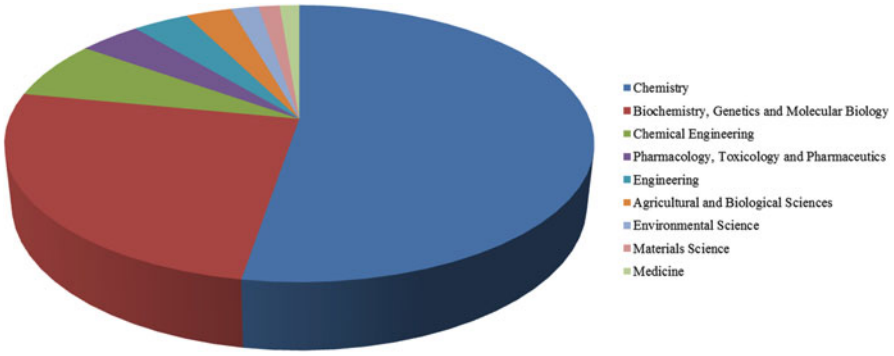

Fig. 7.2 Classification of SFC applications based on number of journal publications on supercritical fluid chromatography from 1967 to 2013. Source: www.scopus.com

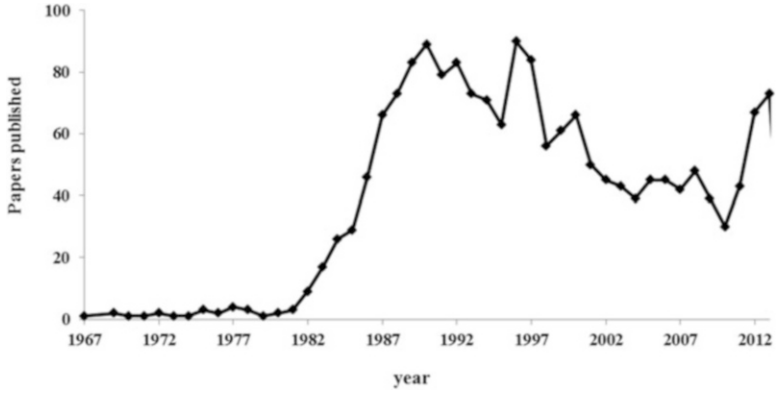

Fig. 7.3 Supercritical fluid chromatography papers published between 1967 and 2013. Source: www.scopus.com

7.1.2 Instrumental Development/SFC Instrumentation

Instrumentation for SFC can be obtained commercially or by adapting systems used for either LC or GC. An SFC system generally consists of a solvent supply, cooling system, pump(s), sample injector, oven or other means of temperature control, column(s), detector, back pressure regulator/restrictor, sample collection and computer to control the entire process. A semi-preparative system constructed and used by the authors is presented in Fig. 7.4. The setup was designed and built in-house and a UV–Vis detector was employed for sample detection and fractionation.

In this section, an overview of current practice for the key components of an SFC system, including mobile phase, stationary phase and detectors is given.

Mobile Phase. SFC pioneers explored a wide range of compounds for use as a mobile phase. During the 1960s and 1970s, dichlorodifluoromethane,

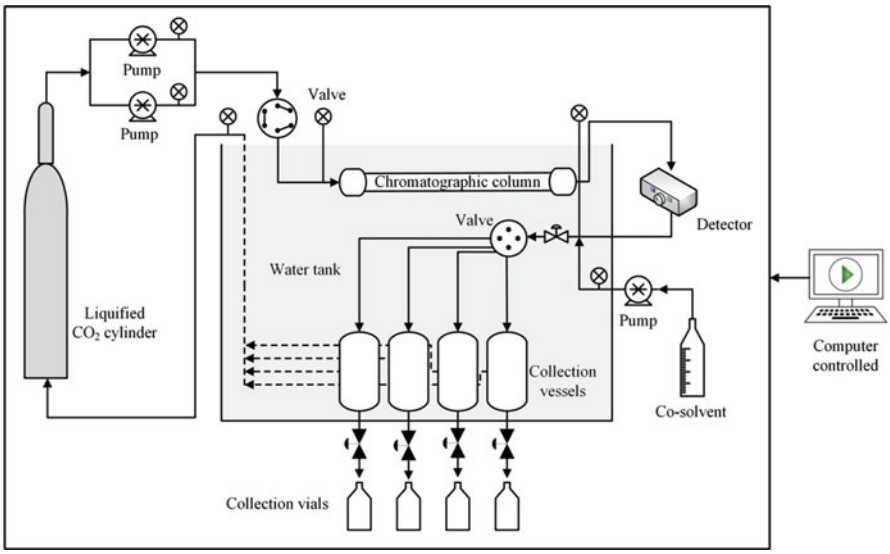

Fig. 7.4 Schematic diagram of a semi-preparative SFC research apparatus

monochlorodifluoromethane, helium, nitrogen, CO_2, and ammonia were employed (Klesper et al. 1962; Karayannis et al. 1968; Giddings et al. 1969). From the late 1970s, SFC focused on CO_2, for its greater convenience, made limited use of organic solvent modifiers, and placed considerable emphasis on the role of pressure and mobile phase density. Today, CO_2 remains the most popular mobile phase because of its low critical temperature, non-toxicity and flammability, and low price compared with other solvents.

Other compounds like nitrous oxide (N_2O) and ammonia are also used, but in a reduced number of applications compared with CO_2. Use of these gases has additional challenges due to their chemical reactivity and toxicity (Raynie 1993). Nitrous oxide has attractive properties as a SFC solvent because its critical properties are similar to those of CO_2 and it has a dipole moment that makes it suitable as a solvent for a wider range of organic compounds. However, its mixtures with organic compounds are potentially explosive and some serious accidents are reported in the literature (Raynie 1993; Sievers and Hansen 1991), making its use in preparative SFC very limited. Argon and xenon have been used as solvents under supercritical fluid conditions and have advantages when infra-red absorption is used for detection, since they are monoatomic (Guiochon and Tarafder 2011).

Supercritical CO_2 is non-polar, similar in solvent properties to hexane, and its elution strength is too weak to elute most polar compounds. Its solvent strength can be increased by adding small volumes of polar organic solvents or increasing pressure (King 2013). Use of these modifiers can influence the SFC separation by changing analyte interaction between the, mobile phase and stationary phase or by competitive absorption on the mobile phase. Most commonly used modifiers are hydrocarbons (propane, cyclohexane, and benzene) but limited due to flammability;

low polarity organic solvents like dichloromethane, ethyl acetate, acetone, tetrahydrofuran, and diisopropyl ether; and polar solvents commonly used in HPLC, like methanol, 2-propanol, ethanol and acetonitrile for separation of polynuclear aromatics and polymers. The use of mixtures of ethanol and CO_2 (50–75 % CO_2) has been recommended by Dos Santos (dos Santos et al. 2009; Pereira et al. 2010) to replace aqueous solutions of acetonitrile for the separation of e.g. nucleobases. All these solvents can be used at temperatures up to ca. 100 °C or higher, but thermal stability of the solutes may place practical limits on their use for some applications.

Water mixed with CO_2 has been used at high temperature. The surface tension of water decreases rapidly with increasing temperature beyond 150 °C, causing a much higher solubility of organics in water. The additions of polar modifiers to supercritical CO_2 was pioneered in the very late 1980s (Ashraf-Khorassani et al. 1988; Berger and Deye 1991a, b; Berger et al. 1989; Steuer et al. 1988), which dramatically increased the polarity of compounds that could be separated by SFC (Berger and Deye 1991c, d). These separations are really subcritical. Many classes of polar solutes including phenols, polyhydroxy, hydroxyacids, polyacids, aliphatic amines, and many drug families were able to be separated using additives like citric acid, trifluoroacetic acid, isopropylamine, triethylamine, ammonium acetate, and many others.

New developments in mobile phase selection include enhanced fluidity mobile phases and switchable solvents. Enhanced fluidity mobile phases used with CO_2 are organic solvents, used conventionally in HPLC, with concentrations higher than 50 %. The role of CO_2 role is to enhance the fluidity of the eluent and increase the rate of diffusion. Examples of solvents employed are fluoroform, or mixture of ethanol with an aqueous buffer of ammonium formate at pH = 3. The major advantages of enhanced-fluidity chromatography (Pereira et al. 2010; Olesik 2004, 2008; Sandra et al. 1995) are (1) eluent viscosity is at least one order of magnitude lower than that of common HPLC solvents, permitting elution at higher mobile phases velocities, the use of longer columns, and/or of columns packed with finer particles; (2) the diffusion coefficients are larger, intermediate between those found in HPLC and in SFC, resulting in a markedly higher optimum velocity and lower mass transfer resistances than in HPLC; and (3) the separations performed are generally better and always faster, particularly in chiral chromatography and in normal phase chromatography. As a drawback, solvent use is higher than is typical for SFC.

A switchable solvent concept has been developed by Jessop et al. (2005, 2010), Mercer and Jessop (2010), in which solvents can exist in two different states, being miscible under one and immiscible under the other. Most such pairs involve changes in the concentration of CO_2 in the system. Examples of switchable pairs of solvents include water and an organic solvent containing R-C(=NR)-NR_2 with R = C_4H_9, which are immiscible but become miscible in the presence of a large concentration of CO_2, the amino group in = NR becoming a quaternary ammonium; and an aqueous solution of tetrahydrofuran and bis-dimethyldiaza (Taylor 2009a; Lesellier 1999) hexane, which separates into an aqueous solution of bicarbonate and the quaternary ammonium of the base and an aqueous solution of

tetrahydrofuran. The advantages of these pairs of solvents are the ease with which a diphasic system can be turned into a monophasic one by changing the concentration of CO_2 in the system. The use of such solvent mixtures could facilitate the separation of the eluent and the components purified by SFC.

Stationary Phase. During the first years of SFC, columns were not designed specifically for SFC operation, instead HPLC and GC columns were used. Commercial SFC column manufacturing didn't become popular until the market grew large enough to justify investment. Early in the twenty-first century, when there was a perception that SFC could be used for separation of a wide range of small-drug molecules, new column development became focused on development of special purpose SFC columns (Majors 2004).

Stationary phases can be easily adapted for use in SFC (Chester et al. 1996). For capillary columns, stationary phases often employed are polymers similar to those used in GC (methyl-, ethyl-, phenyl- and cyanopropyl-polysiloxane and Carbowax (polyethylene glycol) modified silica). Packed columns use porous silica or alumina filled with silica for a polar or moderately polar analysis. SFC stationary phases must be insoluble in the mobile phase used, or they must be cross linked to the silica or alumina. Adsorbents such as porous silica, alumina, titanium, carbon, or molecular sieves have been used successfully. The most common packing materials are those used in HPLC, chemically bonded porous silica. Because the viscosity of SFC mobile phases is low, fine particles can easily be used without requiring high inlet pressures (Berger et al. 2010).

Currently, all companies manufacturing SFC packed columns are offering a similar catalogue of products: traditional amino, cyano, diol and silica phases, and newer options including high-load diol (Diol HL), 2- and 4-ethylpyridine, pyridine amide, diethylaminopropyl (DEAP), propyl acetamide (PA), nitro, pentafluorophenyl (PFP), amino phenyl, or fluoro phenyl. Some of these stationary phases are represented in Fig. 7.5. All these commercial columns are available for analytical or preparative purposes. 2-ethylpyridine was possibly the first stationary phase specifically designed for packed column SFC in 2001. The introduction of this phase initiated a fairly consistent, progressive development of a number of phases with the intent to decrease tailing and provide alternative selectivities. Stationary phases are also available in a range of particle size, from (1.8 to 20) μm; column length ranging from (1 to 100) cm; and column diameter from (0.5 to 100) mm. Capillary column typical dimensions are: (1) film thickness (0.1 to 3) μm; (2) column diameter (0.025 to 0.1) mm; and (3) length (1 to 35) m.

New approaches to stationary phases include hydrophilic interaction liquid chromatography (HILIC) SFC columns (Emmett et al. 2006), solid-core (superficially porous) particles coated with a thick porous layer of silica proposed by Gritti et al. (2010), and use of longer columns. In some circumstances, the most effective means of resolving complex mixtures or difficult to separate pairs is simply to increase the effective number of plates by increasing the column length. The low viscosity and high diffusivity of supercritical fluids allows the use of longer columns with modest pressure drops. Sandra et al. (1994), and Phinney et al. (1998) extended the concept by connecting a series of up to five different

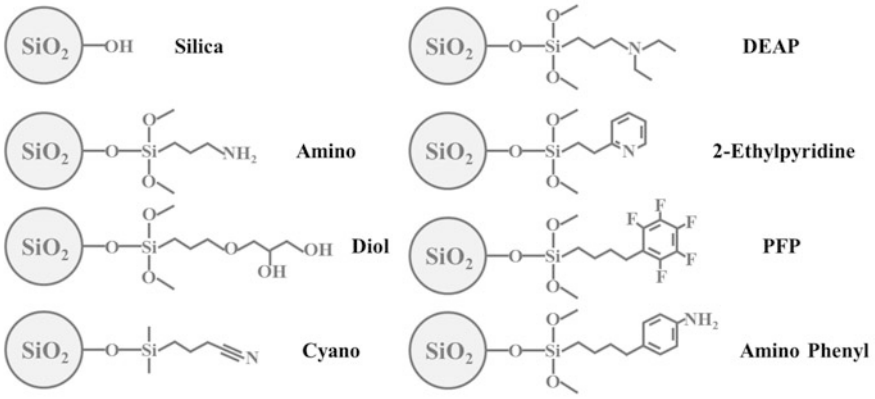

Fig. 7.5 Commercially available stationary phases for SFC packed columns

chiral phases in series, to create a pseudo-universal chiral separation column, or combination of achiral and chiral columns to adjust selectivity in mixtures. Recently, the concept was revived when five 25-cm-long cyano columns were connected in series to provide a high-resolution separation in complex pharmaceutical analysis (Brunelli et al. 2008).

Due to the finite body of knowledge of the interaction between analytes and SFC systems, it is difficult to select most appropriate stationary phase a-priori. In practice, multiple columns need to be examined in order to identify the most suitable stationary phase. To try to overcome this situation West and Lesellier developed a method to classify stationary phases, after having developed a new method of characterising the stationary phases used in SFC (Guiochon and Tarafder 2011). The method is based on the use of the linear solvation energy relationship (LSER) (West and Lesellier 2006a, b, c, 2007). West and Lesellier suggested an original unified classification of stationary phases, which they developed based on a large set of experimental results (West and Lesellier 2008). The classification developed by West and Lesellier is a five-dimensional classification that is based on the five coefficients of the LSER model that the authors had calculated for a hundred solutes and 28 stationary phases, divided into three groups, nonpolar (West and Lesellier 2006c), polar (West and Lesellier 2006b), and aromatic (West and Lesellier 2006a). This exhaustive classification helps in choosing the stationary phase best suited to perform a new separation.

Although open tubular columns similar to those used in gas chromatography have been used in SFC and have permitted the achievement of numerous difficult analyses (Fjeldsted and Lee 1984; Novotny et al. 1971; Peaden et al. 1982), this approach is now less common and there are more similarities between SFC and HPLC columns than between SFC and GC. The considerable advantages of open tubular columns in GC do not transfer well to HPLC nor to SFC (Gere 1982).

Table 7.1 shows different levels of production depending on the column size (I.D.) (Jusforgues and Shaimi 1998):

Table 7.1 Production level

I.D. (mm)	Sample load (gr/h)	Production (ton/year)	CO_2 flow rate (kg/h)
100	60	0.5	100–200
200	240	2	400–800
300	540	4.5	900–1,800
500	1,500	12.5	2,500–5,000

Detectors. SFC can take advantage of most of the types of atmospheric pressure detectors commonly used for both HPLC and GC, although electrochemical detection is not usually applied. SFC can be coupled to mass spectrometers relatively easily. Advances in the detection method have also contributed to the renaissance of SFC. It is possible to use GC and LC type detectors, such as UV–Vis detectors, evaporative light scattering detectors (ELSD) and the most recently incorporated mass spectrometer detector (MS). However, there are few detector options for detection at high pressure for SFC involving recycle of pressurized mobile phase, with UV–Vis being the only commercially available option.

In the 1980s, SFC was used with capillary columns and a flame ionization detector (FID) was the standard detector in open tubular capillary SFC. Today, packed columns and LC-type detectors are frequently employed, as this configuration is most robust and easily applicable to a wide range of analytes.

FID has not been used in packed-column SFC. The stationary phase interactions are typically much stronger than in open tubular capillary SFC; therefore, the addition of a polar modifier is often necessary to elute a sample solute. Even a small amount of organic modifier interferes with an FID, however, FID is still used with packed columns for specific analyses when the mobile phase contains only carbon dioxide, water, argon, or even nitrous oxide. For example, FID is employed in analyses of petroleum fuels using pure CO_2 as the mobile phase. These methods are published by ASTM (American Society for Testing and Materials) as D5186 (ASTM Standard D5186-03 2009) and D6550 (ASTM Standard D6550-10 2003).

Optical detectors such as the UV–Vis spectrophotometric detector, the diode-array UV detector, infra-red spectrophotometric detectors or the refractive index detector require the availability of a cell that is transparent to light and able to operate under pressures of at least 400 bar. These cells are commercially available. The mass spectrometric detector requires a suitable expansion chamber. These detectors can be used with most mixtures of sub- or supercritical fluids and organic modifiers, but it is more difficult to perform trace analysis because SFC provides less sensitive detection and relatively large signal noise.

Evaporative light scattering detection (ESLD) is regarded as a pseudo-universal detector in HPLC and SFC, although photodiode array UV detection (PDA) has become the standard method in packed-column SFC. Since ESLD does not require analytes to have UV absorption, it is a preferred detector in SFC in place of a refractive index detector that is not compatible with the high-backpressure required by SFC. An evaporative light scattering detector (ELSD) should be easier to use in SFC than in HPLC due to the ease of vaporization of the mobile phase. Numerous

applications of ELSD have been reported (Chester and Pinkston 2004). Carbohydrates and various lipids separated by SFC were also detected with ELSD. The major problem encountered in the use of ELSD in SFC is phase separation of the eluent in the tube connecting the column and the detector, where expansion of the eluent takes place (Chester and Pinkston 1998), and the non-linear response of the detector to concentration.

Practical applications of packed column SFC-MS started in the 1990s after successful interfacing with atmospheric pressure ionization, i.e., atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (Huang et al. 1990). MS detection is now a very powerful and indispensable method to identify the target compound accurately, especially in the pharmaceutical industry (Hopfgartner and Bourgogne 2003). A variety of mass spectrometers have been used as detectors in SFC, e.g. quadrupole and time of flight instruments. Because the outlet pressure of an SFC column is not always constant, particularly in the cases of pressure or flow rate programming, the split ratio of the mobile phase does not remain constant when the eluent is split and sent to two different detectors. This might cause difficulties with mass-flow sensitive detectors, like MS. Similarly to LC–MS, APCI and ESI are the most popular ionization methods for the SFC-MS system, allowing the direct introduction of the effluent into the inlet of the mass spectrometer. Although APCI can offer high flow-rates and thus can be considered the best choice of ionization for SFC-MS, very successful applications employing ESI have been emerging in the last few years. Single quadrupole is the commonly used mass analyzer, but also ion trap, time-of-flight (TOF) and triple quadrupole have been coupled to SFC (Hoffman and Taylor 2002; Jentoft and Gouw 1976); in fact, there are no limitations on the mass (Li and Hsieh 2008; Uchikata et al. 2012) analyzer that can be coupled to SFC.

Other components. In modern instruments, reciprocating pumps are often preferred to syringe pumps to compress and deliver CO_2 (Tarafder and Guiochon 2013). These pumps provide accurate mixing for compressible fluids and liquids. Reciprocating pumps eliminate the necessity to refilling syringe pumps (Nathan and Hackbarth 1994), although this inconvenience can be overcome using to syringe pumps in parallel. Operation of reciprocating pumps in certain commercial instruments may lead to a significant instability of CO_2 flow rate. Berger (Berger 1995) and Tarafder (Tarafder and Guiochon 2013) discussed this issue along with the possible problems encountered when pumping compressible fluids like CO_2. CO_2 needs to be chilled before leaving the pump (Sanagi and Smith 1988) and then is pumped in liquid state before being preheated before enter in the column. Pump controllers have algorithms to calculate nominal compressibility compensation (Berger 1995).

In an SFC system, the most important device may be the backpressure regulator which allows pressure control independent of mobile phase flow rate. Saito et al. (1988) developed an electronically controlled backpressure regulator that had a very small internal volume and allowed efficient fractionation without cross contamination between fractions. This type of backpressure regulator has become the standard device in packed-column SFC. Fractions could be collected and stored

under pressure, then gradually depressurized, in which case collection could be complete if the decompression is made slowly enough (Jentoft and Gouw 1972). This is time consuming, but difficult to avoid because flash vaporization of liquid carbon dioxide is endothermic and generates aerosol mists that may carry away part of the products. Rapid depressurization leads to the freezing of a large fraction of the eluent or and product. Losses of up to 25 % were observed by Heaton et al. during their research (Heaton et al. 1996).

The type of injector used to load samples is also important. Injectors can be classified in three different groups: (1) loop injectors, where a low pressure pump is needed to fill the loop. They are used for preliminary tests about column performance and elution parameters; (2) in-line injection: requires a high pressure pump to inject sample, but it is flexible for loading different sample volumes; and (3) in-column injection: to load sample directly onto the column when no dilution is required.

7.2 Process Modelling and System Characterization

7.2.1 Mass Transfer in SFC

Understanding the migration of bands and the progressive evolution of their shape is more complex in SFC due to the compressible nature of the mobile phase. Density and viscosity are highly dependant on pressure. The efficiency of a chromatography column is determined by the mass transfer that occurs within it, and the key parameter controlling the mass transfer is the molecular diffusivity of the compounds to be separated. The diffusivity is often characterised by use of a measured diffusion coefficient of the solute under very dilute conditions which is a measure of the rate at which a solute moves randomly from a region of high concentration to one of lower concentration. There are five steps involved in the mass transfer mechanism in chromatography: (1) axial diffusion along the column; (2) eddy diffusion; (3) external mass transfer through the interface between the flowing mobile phase and the stagnant mobile phase in the particles; (4) diffusion through particles (including pore and surfaced diffusion); and (5) adsorption-desorption. The diffusion coefficient directly controls the kinetics of the first 4 steps, hence its great importance. A more detailed description of diffusion coefficients in SFC and how to measure them is available in the literature (Guiochon and Tarafder 2011; Funazukuri et al. 2004).

7.2.2 *Thermodynamics in SFC*

Unlike in HPLC, equations of state (EoS) play an important role in SFC due to the compressibility of the mobile phase. EoS are thermodynamic equations that mathematically connect the pressure, volume, temperature and other physical properties of a given fluid. In GC, the EoS used is simple as common carrier gases behave as ideal gases. In SFC, however, the EoS becomes crucial as the retention behaviour is strongly related to the density of the mobile phase, which is provided by the EoS as a function of temperature and pressure. Many EoSs with varying degrees of accuracy and ranges of application have been proposed in the literature, but the most commonly used in SFC are the EoS of Peng-Robinson, Lee-Kessler, Span-Wagner, and Saeki (Guiochon and Tarafder 2011). However, there is no general consensus yet as to what the best EoS is for SFC. Span-Wagner, an empirical EoS, is the most accurate but also the most complex equation, and is only valid for pure CO_2. The simpler Lee-Kessler and Peng-Robinson equations can often yield results that are sufficiently accurate for the needs of SFC applications.

The degree of complexity of the EoS needed in a particular SFC system will be determined by how close to the critical point of CO_2 the experimental conditions are, and how large the differences between the sizes and the molecular interactions of CO_2 and of the compounds to be separated are. The addition of a modifier to the mobile phase will further increase this complexity, since the critical conditions of the CO_2 + modifier mixture are not always known under the chosen experimental conditions.

A review of recent findings in this area, along with a discussion of instrumental requirements for thermodynamic measurements, can be found in the literature (Roth 2004).

7.2.3 *Normal* vs *Reverse Phase*

In SFC, reverse phase behaviour can be obtained by using alkyl phases (Gaudin et al. 2000; Lesellier et al. 1999; Lesellier and Tchapla 1999, 2000; Morin et al. 1987; Nomura et al. 1989; Smith and Sanagi 1990), while polar phases can be used for normal phase separations. Phases of intermediate polarity do not necessarily follow either of these modes. Normal phase behaviour is generally assumed based on the idea that the polar modifier adsorbs on the stationary phase in such manner that the stationary phase, covered with modifier, is more polar than the mobile phase (Sandra et al. 1997), and this suggests that all polar solutes should interact more strongly with the stationary phase, whatever this may be, than with the mobile phase. Additionally, the addition of a polar modifier such as methanol decreases retention, which is consistent with a normal phase operation. However, some studies (Gurdale et al. 1999; Lesellier et al. 1993; Lesellier and Tchapla 1998) have shown that, for large and moderately polar compounds, retention decreases

with small amounts of modifier and then increases when the proportion of modifier in the mobile phase is further increased. It has also been shown that the selectivity differences observed between the different stationary phases are essentially due to the stationary phase itself, and not to the silica support or to differential adsorption of the mobile phase components (West and Lesellier 2008).

The traditional distinction between normal and reverse phase behaviour described in liquid chromatography is caused by the different polarities of stationary phases and the necessary change in mobile phase required when working with liquids. However, this frontier does not exist when working with sub- or supercritical fluids and this becomes a somewhat oversimplified classification.

7.2.4 Adsorption Isotherms

Adsorption isotherms describe the distribution of the solute between the mobile and stationary phases, and are the key thermodynamic property describing retention of the solute. Partitioning will depend on the chemical potential of the component in the two phases. The mobile phase in SFC consists of supercritical CO_2, modifier and solutes. The solute concentration is typically low enough that interactions between them can be ignored, and therefore the chemical potential of the mobile phase will only be determined by density, temperature and modifier composition. In the stationary phase, the solid has a finite capacity and all the species -CO_2, modifier and the solutes- compete for adsorption, and quantification of the solubility and the competitive adsorption is required in order to describe the adsorption of the solute under these conditions.

Methods for measuring adsorption isotherms are traditionally classified as static and dynamic (Guiochon and Tarafder 2011; Guiochon et al. 2006). Methods for measuring single-component isotherms can be easily found in the literature (Guiochon and Tarafder 2011; Seidel-Morgenstern 2004). When dealing with multi-component systems in SFC, note that the adsorption of CO_2 and modifier are only considered in an empirical way due to the inherent complexity associated with measuring the competitive adsorption of all components.

Static Methods. In the static method there is no flow involved and the general procedure is to first contact the stationary and mobile phases, add a known quantity of the solute and monitor its concentration either destructively (by sampling a small volume of the fluid phase and analysing it externally) or non-destructively (by using an online detector). When using a destructive method, sampling needs to be carried out carefully in order to not disturb the fluid phase from achieving equilibrium. Furthermore, special care should be taken when injecting the sample into the analytical device (HPLC/SFC), as it could precipitate due to either a pressure drop or a decreased solubility when being injected into another mobile phase. It is important to avoid such precipitation as it might lead to distorted peaks or even to the incorrect estimation of the sample amount. Non-destructive techniques can eliminate these problems, i.e. by incorporating a UV online detector within the

measurement system and circulating the fluid using a pump (Su et al. 2009). Although this approach provides a good method to obtain equilibrium information, dead volume effects may have significant impact on the reliability of the data and measuring kinetics needs to be performed with caution. The main advantage of static methods is the possibility to estimate adsorption data up to high concentrations without requiring significant amounts of material, but they are generally considered cumbersome and time-consuming.

Dynamic Methods. Dynamic methods, known also as chromatographic methods, offer higher speed and accuracy over static methods, and involve the use of standard HPLC and SFC equipment for the measurement of isotherm parameters. The various dynamic methods differ either in the type of disturbance provided or the method in which the response is analysed (Guiochon and Tarafder 2011). One of the most important methods is the inverse chromatography method, in which the elution band profile of the multi-component sample calculated with an assumed set of competitive isotherms is compared with the experimental band profile and the isotherm coefficients are adjusted to minimize the difference. Another typical dynamic measurement is to cause a known disturbance at the column inlet and then measure the response of the column using a detector.

Estimation of Non-Linear Isotherms. Most preparative separations involve overloading the column with fairly high concentrations of the solute. Under these conditions, due to the limited capacity of the solid phase, the adsorption isotherm is non-linear. There are several methods described in the literature to measure non-linear isotherms (Guiochon et al. 2006; Seidel-Morgenstern 2004); a few will be discussed here. The bed voidage, ε, and the Henry constant, H, need to be measured first in order to measure non-linear isotherms. The void volume of the bed can be obtained by measuring the retention time of a non-adsorbing tracer, and it is recommended that this is performed at the highest possible modifier composition in order to minimise the possible adsorption of the tracer. The Henry constant can be measured by injecting a very dilute mixture of the solute in the mobile phase. The retention time obtained can then be used to obtain the Henry constant according to Eq. (7.1), where L, v and ε refer to the column length, the interstitial velocity and the bed voidage, respectively. Due to the effects of compressibility, all measurements should be performed at flow rates where the density gradient across the column is minimal (<5 %).

$$t_{R,i} = \frac{L}{v}\left(1 + \frac{1-\varepsilon}{\varepsilon} H_i\right) \tag{7.1}$$

Inverse Method. In the inverse method, a known concentration and volume of the solute mixture is injected and the chromatogram is measured (Felinger et al. 2003; Rajendran 2012). For improved reliability, other injections either with different injection volumes or concentrations are made. A careful calibration of the detector is required in this method, as often the calibration curve is not linear. As discussed above, this method relies on the fact that when the appropriate isotherm and mass

transfer models along with the correct parameters are used, it will be possible to calculate the band profiles; hence, an isotherm and mass transfer model are initially assumed.

The form of the isotherm is predicted from the chromatograms themselves. Some isotherm models directly reflect the competitive effects (competitive Langmuir, competitive bi-Langmuir, generalized Langmuir, etc.), but when this is not possible models which allow combining single-component isotherms to describe binary adsorption, such as the ideal adsorbed solution (IAS) theory, are employed. Once the isotherm function and a framework to describe the adsorption are chosen, these data are incorporated into a simulation model to obtain the calculated elution profile. The parameters of the model are then optimised in such a way that the error between the calculated and measured elution profiles is minimized, and these optimised values of the parameters are considered to describe the isotherm. This method can be applied to systems characterized by any form of isotherm, and it allows combining multiple experiments to obtain a set of values that describe a wide range of concentrations. However, it is not suited to obtaining reliable parameters at high concentrations, as the pulse will suffer a dilution as it travels through the column and the measured isotherms are strictly valid only up to the maximum concentration measured at the column outlet.

Perturbation Method. The perturbation method consists of saturating the column with a known concentration by continuously pumping the solute in the mobile phase before introducing a small disturbance at the column inlet and measuring the response of the column using a detector (Rajendran 2012). This method requires large amounts of solute, but its implementation is straight-forward, no detector calibration is needed and it allows characterization at very high concentrations as there are no dilution effect as in the case of the inverse method described above.

Binary Retention Time Method. This method is based on the principle that the retention time of the discontinuity formed by injecting a high-concentration pulse can be calculated by equilibrium theory of chromatography (Rajendran and Chen 2009). The experiments required for this method are the same as for the inverse method, but only the retention time of the two shock fronts is extracted from the chromatograms. These retention times are also estimated with the equilibrium theory for competitive Langmuir isotherms for the given injection concentration and volume with assumed Langmuir parameters. These parameters are then optimised by minimising the error between experimental and estimated retention times. The main advantages of this technique over the inverse method is that no detector calibration is required, since only retention times are measured, and that the retention times can be estimated using rather simple analytical equations, which considerably shortens the time required. However, this method is based on competitive Langmuir isotherms, so applying it to non-Langmuir systems could result in misleading values. In systems with significant mass transfer effects this method could also produce inaccurate results. Nevertheless, this method can be used to obtain good initial estimates for more rigorous methods such as the inverse method.

7.2.5 Pressure Drop

In order to avoid undesirable band-broadening and even damage to the stationary phase, the pressure drop limit set by the column manufacturer should be observed. It is therefore recommended to characterise the pressure drop of columns before further operation using Darcy's equation, i.e. Eq. (7.2), where ρ is the local density of the mobile phase, u its interstitial velocity, μ its viscosity and β an empirical parameter depending on the characteristics of the column used.

$$\frac{dP}{dz} = -\beta \frac{(\rho u)(\mu)}{\rho} \tag{7.2}$$

In principle, a single experiment at a moderate flow rate in the absence of solute would be sufficient to estimate β for a given column, and it would only need further validation if the stationary phase is suspected to have been significantly altered. Once β has been determined experimentally at a single point, the pressure drop at other conditions can be easily estimated.

In GC, the pressure drop tends to be small, but it results in a large change in density that has little effect on retention. In HPLC, pressure drops are typically large, but the change in density is negligible due to the incompressible nature of the mobile phase. Rajendran et al. (2005) examined the case of SFC where density changes within the column have an impact on separation efficiency. They found the pressure drop to be much lower than what is typically reported in HPLC, and that much of the efficiency loss in this density region was related to band spreading due to axial and radial temperature gradients arising from Joule-Thompson cooling of the fluid flowing through the columns.

7.2.6 Mobile Phase Flow Rate

The flow rate of the mobile phase will affect the column efficiency and the separations achieved. However, in most recent publications on SFC, especially those reporting analytical applications, the information provided on the experimental conditions are generally insufficient. Both the mass and the volumetric flow rates of the mobile phase need to be known when analysing the results of an SFC operation. The two main physical characteristics that control the separation power in chromatography are (a) the mass transfer properties of solute molecules through the mobile phase that permeates the column, and (b) the thermodynamic interactions between the solute molecules and the stationary phase. Inside a chromatographic column, the transport properties of solute molecules depend on the interstitial velocity of the mobile phase, which in turn is related to its volumetric flow rate. In contrast, the retention factors and other thermodynamic properties depend on the mobile phase density and composition, which are controlled by the

individual mass flow rates of the mobile phase components (Guiochon and Tarafder 2011; Tarafder and Guiochon 2013).

While early publications provided detailed information on the method used for flow rate measurements, more recent publications have become less detailed. In some cases, a flow rate value is provided but the measuring technique used is not given (van Wasen and Schneider 1975). In other publications authors have used soap bubble flow meters in which the mobile phase is expanded by a two-stage reducing valve placed downstream the detector (Wilsch et al. 1983), a thermal mass flow meter placed in an ambient pressure flow path downstream of the backpressure regulator (Gere 1982), or a mass-flow controller and a mass-flow meter placed in series at the outlet of the chromatograph downstream of the detector (Schoenmakers and Uunk 1987). Using a thermal mass flow meter requires knowledge of the thermal properties of the fluid, and in the case of mixtures these are rarely known with enough accuracy for this purpose. Some authors have reported using a syringe pump with a cooled head and estimating the mass flow rate based on the volumetric flow rate delivered by the pump, the temperature and the pressure of CO_2 at the pump head (Rajendran et al. 2005; Poe and Schroden 2009). The accuracy of the mass flow rates estimated in these studies depends heavily on the accuracy of the parameters provided by the pump. Among more recent publications, detailed information on the measurement and control of the flow rates is mainly found in those of academic origin.

7.3 Scale-Up

7.3.1 Analytical SFC

The initial development of SFC as an analytical tool was restricted to the use of pure carbon dioxide, and its limited solvation power, which resulted in relatively large retention factors and long analysis times. More recently, due to increased instrument sophistication, SFC is now a genuinely useful separation method, which can provide analysis with performance comparable to that of HPLC. Modern SFC may be performed with a complex CO_2 based mobile phase, comprising multiple modifiers. In terms of analytical applications, SFC separation of alkanes, of cyclic and aromatic hydrocarbons has been thoroughly investigated (Jentoft and Gouw 1976; Novotny et al. 1971, 1981; Peaden et al. 1982; Bartmann and Schneider 1973), and as such is utilised in the pharmaceutical and specialty chemical industries (Zheng et al. 2006). The relative speed with which the organic substrates can be separated from the mobile phase is one of the most important advantages of SFC in the drug discovery process. SFC complements RPLC, particularly for the analysis of highly hydrophobic compounds (Taylor 2009b). A wide variety of applications of SFC to the analysis of foods (e.g. lipids, sterols, polyphenolic compounds or traces of pesticides), natural products, fossil fuels, monomers, oligomers,

polymers and polymer additives, of pharmaceutical intermediates, achiral or chiral (Chester and Pinkston 2004) has been reported. Examples exist whereby analysis is facilitated by coupling on- or off-line supercritical fluid extraction, SFC, and mass spectrometry (Sandra et al. 1995).

Analytical SFC has been applied to a wide variety of materials (Salvador 1996) although the number of publications in food analysis is smaller than in other areas. Applications have primarily focused on the determination of lipid compounds, due to polarity compatibility but are increasingly expanding toward analysis of polar analytes by use of polar additives, including water (Taylor 2012).

7.3.2 Preparative SFC

The design of a preparative separation involves selection of a suitable stationary and mobile phase combination. The screening of suitable phases is typically automated, where a series of analytical columns and modifiers are chosen and various combinations are used. Key properties that are evaluated are the retention characteristics, the selectivity and resolution. Once reasonable values of selectivity and resolution are obtained, a stationary phase-mobile phase combination is chosen for detailed characterization and determination of scale-up conditions.

Characterisation is typically achieved using loading studies, in which pulses overloaded by volume or concentration are injected and responses measured. The volumes and concentrations at which baseline separation is achieved are translated to allow scale-up determination. However, this strategy does not explore the entire envelope of variables within which the separation is feasible, and the specific objectives in a given separation may vary. Characterisation may include measurement of adsorption isotherms, mass transfer kinetics, pressure drop parameters and solubility, which may be performed on analytical-scale. Computer aided simulations may also be performed for assistance in process optimisation via calculation of band profiles and identification of operating conditions that optimize certain objective functions. Such an approach has been extensively covered in the literature (Rathore and Winkle 2009; Yu 2008).

In a manufacturing process, overall cost efficiency may be the key objective. Once operational requirements and process outcomes are established, the specific operating conditions that will guarantee the desired outcome can be defined. A comparison against the effectiveness of different process options may be performed.

7.3.3 Industrial Scale

Information on the design of supercritical fluid equipment and installations is provided by Brunner (Brunner 1994). Economic considerations dictate the

suitability of process implementation. This depends in part on the specific purpose of the separation; for example, a unit build according to current Good Manufacturing Practices (cGMP) requirements will cost (30–100) % more than a similar food-grade unit. The operating costs may also be reduced for a large capacity unit (Guiochon and Tarafder 2011).

The commercial needs of preparative scale separations may influence the further development and application of analytical scale SFC, particularly since analytical separations are often used to model and develop preparative separations. Generally, interest is focussed on pharmaceuticals, natural products, and polymers. Several general articles give perspectives and address capabilities (Barker 2003; Berger et al. 2002; Edwards 2003; Hughes and Hunter 2001), although understandably, few specific industrial applications have been published. Taylor and King (Taylor and King 2002) used preparative supercritical fluid extraction combined with preparative SFC to obtain extract fractions enriched with free sterols and ferulate-phytosterol esters.

7.3.4 Simulated Moving Bed (SMB)

Single column SFC has several advantages as its implementation and scale-up are straightforward and it is well suited to the separation of multi-component mixtures. However, the productivity of a single column is rather limited owing to the fixed-bed configuration. For the purification of large batches, multi-column chromatography (MCC) may offer advantages in terms of productivity and solvent consumption (Juza et al. 2000; Rajendran et al. 2009). One of the most popular implementations of the MCC is simulated moving bed (SMB) chromatography (Nicoud et al. 1993). The main objective in SMB is to overcome the fixed bed operation of the single column chromatography and to implement a configuration in which the stationary and the mobile phases move in counter current directions.

In 1996, Clavier and Nicoud proposed the use of supercritical fluids as a mobile phase for the SMB process (SF-SMB) (Clavier et al. 1996). The group of Johannsen successfully demonstrated the application of SF-SMB in the separation of isomers of phytol (Depta et al. 1999), enantiomers of ibuprofen (Peper et al. 2002), and tocopherols (Lübbert et al. 2007). They were able to obtain high purities for both raffinate and extract products. While control of operating pressure can be used to implement a gradient in retention time, a gradient in modifier composition can also be used to the same effect. This principle has been exploited in liquid SMBs (Abel et al. 2002) but has not been explored for SF-SMB applications. However, rigorous optimization techniques have not been applied for SF-SMB design and the trade-off of implementing pressure and/or modifier gradients must be evaluated. The SF-SMB process, while having significant potential to improve productivity and to reduce organic solvent consumption has not taken-off at the industrial scale. Part of the reason is due to the heavy investment costs associated with the equipment

(Peper et al. 2007). However, if there are needs for large-scale purifications, e.g., tons of product, then it maybe worth considering this option.

7.4 Food and Nutraceutical Applications

Food-related applications of SFC are included in numerous reviews. Analysis and separations of fatty acids and triacylglycerols are extensively reviewed (Rezanka and Votruba 2002; Señoráns and Ibáñez 2002; Soheili et al. 2002), as is triglyceride speciation (Ahmed 2001; Brondz 2002; Ibáñez and Cifuentes 2001), and a variety of literature exists concerning topics such as analysis of pesticides and their metabolites in foodstuffs (Andrikopoulos 2002a, b). The use of open-tubular and packed-column SFC for free fatty acids and fatty acid methyl esters is reported, including discussion of packed-column stationary phases suitability for the elution of free fatty acids with unmodified CO_2 (Señoráns and Ibáñez 2002). It is noteworthy that research has not only been focussed toward purification of different compounds, but also developing the technology by creation of advanced equipment. For example, Alkio et al. (1988) transformed one extraction unit into a SFC preparative unit with column volumes ranging between (0.3–2) litres, using CO_2 as the mobile phase with flow rates greater than 8 kg/h. The versatility of the technology is exemplified by a large number of instances using the strategy of coupling multiple columns in series in order to achieve desired separations.

Glycolipid class profiling of wheat was studied by Lesellier and co-workers, utilising MeOH-modified CO_2 to separate neutral lipids, glycosylated sterols, glucosylceramides, and glycoglycerolipids. This was made possible by coupling silica and diol columns (Deschamps et al. 2004).

It is also logical to couple SFC with SFE, since the classes of compounds to be handled are common, as are aspects of the technologies and expertise. Two publications describe the use of SFC to monitor the products of enzymatic reactions in supercritical CO_2 (King et al. 2001; Rezaei and Temelli 2001). King et al. compared various enzymes for the formation of sterol esters (King et al. 2001), observing over 90 % conversion of cholesterol and sitostanol with C_8 through C_{18} fatty acids. In contrast, Rezaei and Temelli studied the hydrolysis of canola oil triglycerydes by an immobilized lipase *from Mucormiehei* (Rezaei and Temelli 2001), concluding that on-line extraction/reaction was a promising process for producing high-value products from oil seeds. In other examples of SFC applies to food based applications, SFC has been used to characterize polyphenolic compounds in SFE extracts, employing techniques such as using methanol with 1 % citric acid as a mobile-phase modifier and two coupled diol-modified silica columns to achieve separation (Komangerpour et al. 2002). Combined SFE/SFC with on-line GC-MS has been employed to study semi volatile compounds in fresh, stored, raw, and baked Baltic herring (Aro et al. 2002), with the SFE extract fractionated by on-line SFC, and the volatile fraction directed to a GC-MS.

7.4.1 Lipids

Lipids are one of the three principal macronutrients and play a vital role in many biological processes such as energy storage or cellular signalling; they are also structural components of cell membranes (Hermansson et al. 2005; Wenk 2005). Their diversity is very high due to the different combinations of hydrophobic acyl chains, and their polarity can vary as a consequence of association with hydrophilic moieties such as phosphoric acid (phospholipids) or carbohydrates (glycolipids). In recent years a wide range of lipid focussed analytical methodologies have been established. Of particular significance are those based on chromatographic techniques such as GC (Cruz-Hernández and Destaillats 2012; Purcaro et al. 2010; Ruiz-Samblás et al. 2010), HPLC (Camera et al. 2010; Pang et al. 2008), UPLC (Ikeda et al. 2009) and SFC (Bamba et al. 2012; Lesellier 2001a; Smith et al. 2001), either with or without mass spectrometry. The possibilities and limitations of the LC and GC methods have been discussed in several reviews (Buchgraber et al. 2004; Myher and Kuksis 1995), so here we focus on the implications of SFC based methodologies. As for other product applications, CO_2 is the most established mobile phase in SFC for separation of lipids, providing high resolution per unit of time (if compared with the same column in HPLC). As SFC operates at low to moderate temperature, it is well suited for the analysis of high molecular weight lipids like triacylglycerols. Even though HPLC methods give excellent resolutions, the elution times are relatively long and quantitative detection becomes a problem. With GC there is a possibility of thermal cracking of the stationary phase or of the sample (Christie 1990). SFC has also been applied to analysis of phospholipids after conversion to diacylglycerol derivatives (Sandra and David 1996), biosynthetic polyunsaturated fatty acids (PUFAs) (Wang and Muttucumaru 2002), cholesterol and its esters in human serum (Kim et al. 1994; Nomura et al. 1993) and food samples (Ong et al. 1990), mono-, di- and triglycerides in pharmaceutical excipients (Giron et al. 1992). SFC has also been applied to analysis of archaebacterial lipids and glycosphingolipids (Hayes 1997). Review articles have appeared in recent years on this topic (e.g. (Blomberg et al. 1998; Laakso 1992)) and should be consulted for more detailed information.

A number of methods for fat and oil analysis (Señoráns and Ibáñez 2002; Lesellier 2001b), including for fatty acid methyl esters (FAMEs) and free fatty acids (FFAs), allow origin, type of oil, and quality assessment; extraction and analysis of unsaponificable lipids of high molecular weight and complex structures; and determination of minor compounds in high-valued oils as sterols, tocopherols and carotenoids (Ibáñez et al. 2000).

Fatty Acids. Analysis of fatty acids is important, not only because fatty acids are valuable nutritional substances in living organisms, but because they are also used to characterize the quality of commercial oils. Traditionally, fatty acids have been analysed by means of GC, although their low volatility (especially those of high-molecular weight) and their relatively high polarity, causes peak tailing and requirement for high temperatures. Thus, most GC analyses are based on analysis

of volatile derivatives, usually methyl esters, which provide very efficient separations. HPLC avoids the problems of thermal degradation and historical problems of poor resolution and low sensitivity are negated by use of mass spectrometry detectors. SFC offers the possibility of using low temperatures, high flow-rates, direct injection of the sample diluted in *n*-hexane or *n*-heptane, low volatility lipids such as triglycerides, use of universal detectors such as FID, ELSD or MS, and the simultaneous analysis of fatty acids and other lipid classes (triacylglycerols, cholesterol, etc.). Both open and packed columns have been used in SFC for the separation of fatty acids (such as FFAs or FAMEs), and in most cases the use of an organic modifier is necessary in order to increase the polarity of the mobile phase and to obtain acceptable peak shapes and retention. Considerable efforts have been made to develop new stationary phases in order to overcome these problems. All these studies and their applications to the analysis of fatty acids in foods were reviewed in 2002 by Señoráns and Ibáñez (2002). More recently the use of multidimensional chromatography, in order to improve the separation of very complex mixtures, has been examined in several studies (Dallüge et al. 2003; Jover et al. 2005; Manzano et al. 2011, 2012). Hirata and Sogabe pioneered the use of comprehensive two-dimensional supercritical fluid chromatography (Hirata and Sogabe 2004). They developed a system with conventional packed columns and a FID detector for separating FAMEs from various edible oils. In the first dimension, separations on the silica gel column were determined by the number of double bonds, with higher retention as the number of double bonds increased. In the second dimension, an ODS column provided differentiation of retention based on chain length. This system provided very well ordered chromatograms and enhanced resolutions, allowing the easy detection and identification of minor components. The coupling of SFC and RP-HPLC was studied by François and Sandra (François and Sandra 2009) for separation of derivatized fatty acids (such as phenacyl esters) of fish oil, employing ELSD and UV detectors. In the first dimension, two silver-loaded columns, prepared from strong acidic cation exchange columns, were used and gradients of modifier and system pressure were applied; here, compounds with the same number of double bonds coeluted. In the second dimension, a gradient of water/acetonitrile was applied to a C_{18} column. Guard columns were used as packed loops for trapping the analytes from the SFC effluent in order to focus the analytes before transfer to the second dimension, where compounds were separated according to their chain length.

Today, mass spectrometry detectors are preferred for analysis of complex samples. As such, SFC has been coupled with triple quadrupole mass spectrometry by Hori et al. to determine 3- monochloropropane-1,2-diol (3-MCPD) fatty acid esters in edible oils (Hori et al. 2012a). These are generated in the refining processes of oils and fats, and can cause adverse effects on the kidney, therefore analysis of their presence in edible oils is of interest. In the reported SFC method 14 analytes were separated without sample purification, instead the analytes were detected as ammonium adducts using electrospray ionization (ESI) in the positive ion mode. Detection limits ranged from 0.013 to 0.063 mg/kg. Alternative methods required

hydrolysis and derivatization (GC), because of loss of sensitivity due to peak broadening (HPLC) (Hori et al. 2012b).

Polyunsaturated Fatty Acids. Due to the importance of polyunsaturated fatty acids (PUFAs) in human health, and especially the ω-3 series, there is an increasing effort to produce highly enriched mixtures of eicosapentaenoic acid (EPA, C20:5ω-3) and docosahexaenoic acid (DHA, C22:6ω-3), both of which are deemed high value. These long chain omega-3 fatty acids are essential in the human diet for proper growth, development and good health, cannot be chemically synthesised economically, and it is necessary to recover them from natural sources in order to concentrate them for use in nutritional supplements. Nutrition experts suggest that an omega-6: omega-3 fatty acid ratio of 5:1 or less is desired (WHO/FAO 1994). However, modern food habits in Western countries result in a ratio reaching values of up to 100:1. Marine oils, especially fish oil, provide the major natural dietary source of EPA and DHA. Interest has also turned to algae (e.g. *Phaeoactylum tricornutum*, *Monodus subterraneus*, *Nannocloropsis*) as they are not seasonal products, and do not have either unpleasant odour or a high amount of cholesterol, and can contain squalene and phytosterols as additional beneficial compounds (Conchillo et al. 2006; Kalogeropoulos et al. 2010). However, the cost of the extraction processes are considered high since the desired fatty acids are mainly membrane bound; and production by fermentation is generally prohibitive also. Nevertheless, DHA in the form of triglycerides produced by microalgae is now increasingly used in infant formula (Belkind-Gerson et al. 2008; Hawthorne et al. 2009; Hoffman et al. 2008; Jensen et al. 2005; Kralovec et al. 2012). To obtain a more desirable product in which EPA and DHA are highly concentrated, naturally derived oils must be converted into fatty acid methyl or ethyl esters, or free fatty acids, since PUFAs in the triglyceride form are difficult to concentrate due to the fatty acids being more or less randomly distributed in this form. There are many methods for the fractionation of fatty acid methyl or ethyl esters, including widely used chromatographic methods such as HPLC (Beebe et al. 1988) and silver resin chromatography (Adlof and Emken 1985; Guil-Guerrero and Belarbi 2001; Perrut 1988; Teshima et al. 1978), molecular distillation (Jiang et al. 2006; Rossi et al. 2011), enzymatic splitting (Kojima et al. 2006), urea complexation (Liu et al. 2006) and supercritical fluid extraction/fractionation techniques (Alkio et al. 2000; Higashidate et al. 1990; Nilsson et al. 1988; Perrut et al. 1998; Pettinello et al. 2000; Snoey-Elich 2001). Some concentration of esters or fatty acids is possible by direct fractionation using supercritical CO_2, but only on the basis of chain length and not on the degree of unsaturation. SFC makes this separation possible, combining the selectivity of a solid phase towards double bonds and the selectivity of supercritical CO_2 as a solvent towards the number of carbon atoms. Studies on the separation of PUFAs with SFC are limited, applying reversed phase chromatography (Perrut et al. 1998) or using NP silica based stationary phases (Higashidate et al. 1990). There are some studies using fatty acidethyl esters from different fish oil origins such as sardine, tuna and menhaden (Alkio et al. 2000; Higashidate et al. 1990; Nilsson et al. 1988). Perrut et al. (1998) achieved EPA and DHA fractions with purities of 92 % and 85 %, respectively, and yields of 99 %

starting from a feed material containing 50 % EPA and 30 % DHA. The technique coupled preparative SFC and simulated counter-current moving bed chromatography with several columns placed in series. Higashidate et al. (1990) used a silver nitrate-loaded silica gel column at a laboratory scale to separate fatty acid extracts from the CO_2 extraction of esterified sardine oil (initial EPA and DHA contents of 12 % and 13 %, respectively). Using this method the authors obtained EPA and DHA-rich fractions with purities of 93 % and 82 %, respectively. The changes upon heating of low-linolenic soybean oil versus partially hydrogenated soybean oil were investigated by Soheili et al. (2002). SFC analyses revealed the partially hydrogenated oil to be more stable than the genetically modified low-linolenic oil at a significant level. Nilsson et al. (1988) employed an increasing pressure programme in conjunction with a temperature gradient for fractionation of urea crystallised fish oil ethyl esters (initial EPA, 49 %; DHA, 22 %). With this method they were able to recover more than 85 % of the EPA and DHA from the feed with 90 % purity. Snoey-Elich was also able to concentrate fish oil EPA-EE (ethyl ester) from a purity of (50 to >95) % (Snoey-Elich 2001). This was achieved using an industrial column of porous silicon dioxide impregnated with (3-aminopropyl)-triethoxysilane as the stationary phase, and $SCCO_2$ as mobile phase. The economic feasibility of producing industrial-scale quantities of EPA and DHA using SFC was investigated by Alkio et al. (2000). They reported that it was possible to obtain simultaneously DHA and EPA ethyl esters with purities of 90 % and 50 %, respectively, from a feed already highly enriched in EPA and DHA obtained by urea fractionation. Pettinello et al. (2000) carried out pilot scale trials using quantities of feed materials on the order of hundreds of grams with CO_2 recycling. By optimising the feed loading, temperature and pressure for this process, a 93 % pure EPA rich fraction at 25 % yield was achieved. So far the only known commercial scale production of highly pure ethyl esters is carried out by KD Pharma (Pharma 2013), offering products with EPA purities up to 99 % or DHA purities up to 95 % employing the patented kd-pür™ technology. Montañés et al. carried out a range of optimisation studies at semi-preparative scale to evaluate operating conditions and a range of proprietary columns, for separation of EPA and DHA from marine and algal oils (Montañés et al. 2013), juniperonic acid from *Biota orientalis* seed oil and xymenynic acid from sandalwood seed oil (Catchpole et al. 2012). Triglyceride oils were first converted to free fatty acids or ethyl esters before achieving >95 % separation and good recovery using CO_2 without co-solvent and on modified or unmodified silica. The results displayed a high enrichment of EPA-EE with values up to 99 % and DHA-EE enrichment up to 83 %. For some stationary phases C_{22} purity was close to 100 %. Under the optimal conditions EPA ethyl ester was recovered at almost 100 % purity from fish oil and 97 % purity from algae oil, and DHA ethyl ester at 83 % purity was obtained from fish oil ethyl esters at a yield of around 75 %. Stationary phase particle size was found to be the most important parameter affecting the separation of long chain omega-3 fatty acid/ethyl esters, followed by the packing type, injection volume and the temperature/pressure conditions used for the oils investigated. The particle size is obviously an important consideration for scale up of the process. For pilot/industrial processes with large columns,

significantly increased quantities of stationary phase will be required, giving rise to high costs and high pressure drops for small particle sizes, and reduced cost, pressure drop and separation performance for large particle sizes. More recently, the same group commissioned a preparative chromatography pilot plant (Tallon et al. 2012; Rose et al. 2012), holding up to four 10 L columns, up to 4 separation stages, on-line UV–Vis absorbance measurement and supercritical or liquefied gas mobile phase flow rates up to 100 kg h^{-1}. EPA and DHA peaks were not completely separated in initial trials, but reasonable concentration of EPA was able to be achieved using relatively coarse unmodified silica.

The preparative separation of EPA and DHA using SFC was pioneered by Perrut and Jusforgues (1989). Alkio et al. (2000) used prep-SFC with pure CO_2 and a C_{18} column to purify EPA and DHA ethyl esters from trans-esterified tuna oil. They observed that the production rate was greatly influenced by the load, and when this exceeded a certain value, at constant purity, the production rate decreased as a consequence of the reduction in the purity level of the fractions collected. Under optimal conditions a simultaneous production of 0.85 g DHA ester/(kg stationary phase h) at a purity level of 90 wt%, and 0.23 g EPA ester/(kg stationary phase h) at 50 wt% purity was obtained. EPA ethyl ester was also purified by Pettinello et al. (2000) from a mixture of fish oil. In this case a purity of 95 % and 11 % yield were obtained on a silica gel column. Higher yields (43 %) could be reached but with a lower purity (90 %). Alkio et al. studied also economic feasibility of EPA and DHA concentrates from transesterified tuna oil (Alkio et al. 2000). DHA was obtained with purities higher than 95 % using CO_2 as a mobile phase at 65 °C and 145 bar, with reverse phase octadecyl silica column. DHA and EPA esters can be produced simultaneously with purities up to 90 % and 50 %, respectively. The authors' estimation was that 160 kg of stationary phase and 2.6 t/h of recirculated CO_2 is needed to produce 1,000 kg of concentrated DHA and 410 kg of concentrated EPA at an operational cost of US$550/kg of concentrated ethyl ester. Yamaguchi et al. developed a silver coated silica stationary phase, to purify DHA-EE from fish oil using SFC at pilot scale, achieving purities up to 95 % (Yamaguchi et al. 1999).

With respect to adsorption-desorption processes using SFC technology, some useful studies have been performed towards fats and oils treatments (McLachlan et al. 1991a, b), regarding selective removal of cholesterol from mixtures with CO_2.

Further research has been conducted to obtain highly concentrated EPA and DHA (Létisse and Comeau 2008; Weber et al. 2008) and γ-Linolenic acid (GLA) (Venskutonis et al. 2008) extracts. The main difficulty for polyunsaturated fatty acids enrichment is the complexity of the material, with triglycerides esterified with different chain length and saturation of the ligands.

At preparative scale, several key applications have been described. Taylor and King (2002) developed a coupled SFE/SFC process to extract and purify free sterols and phytosterol esters from maize bran. The authors suggested that the process could be used at industrial scale to produce functional ingredients. Taylor also developed the extraction and purification of phospholipids from soy flakes. The sample was defatted with CO_2 and later phospholipids were extracted with ethanol

with an alumina column and using an ethanol/water gradient (Taylor et al. 2000). Sesti-Osseo et al. studied continuous peanut oil fractionation on a packed column and CO_2, showing that SFC technology can be effective to fractionate oil compounds according to polarity and molecular weight, achieving selective recovery of polymeric compounds, triglycerides and low molecular weight compounds (Sesti-Osseo et al. 2003). Yuen May Choo et al. employed a method for purification of carotenoids from palm oil using preparative SFC (Choo et al. 1996). Separation of tocopherol isomers using SFC, has been also studied at analytical scale using a silica column and CO_2 with alcohol (Johannsen and Brunner 2003). The same authors developed a method for mixtures of tocopherols and tocotrienols fractionation using a Kromasil column and CO_2 + isopropanol as mobile phase (Peper et al. 2003).

Triacylglycerols. Triacylglycerols (TAGs) are the most prevalent natural form of neutral lipids. They are fatty acid esters of glycerol, and exist within organisms as a source of energy and essential FAs. Dietary imbalance can contribute to several diseases such as coronary heart disease, dyslipidaemia and obesity or inborn metabolism disorders. In the human diet the main source of TAGs are vegetable oils and animal fats. Determining TAGs is important and has been proposed to assess milk fat purity (Commission E 1995). Considering the diversity of fatty acids that can esterify glycerol and the large number of combinations, it is clear that there is a wide variety of TAGs and so their determination is a difficult undertaking. The chromatographic separation of triacylglycerols most typically involves high-performance liquid chromatography (HPLC) with reversed-phase or silver-ion columns (Dugo et al. 2004; Laakso and Voutilainen 1996; Morera Pons et al. 1998). In addition, gas chromatography (GC) has been used to separate the methyl esters obtained by trans-esterification of the TAGs (Fontecha et al. 2005; Park et al. 2010). However, this option has several problems due to the fact that the transesterification of polyunsaturated triacylglycerols is not always quantitative (Milinsk et al. 2008; Shantha and Napolitano 1992) and that there is a lack of information concerning intact TAG composition. Direct GC analysis is very difficult because of the low volatility of these compounds and the high temperatures required, although this technique is used in several validated methods. Triacylglycerols have maximum absorbance at very low wavelengths, so the use of UV detectors in TAG analyses is very limited. ELSD is most commonly used, although mass spectrometry is considered preferable where structural elucidation is required (Dugo et al. 2004; Laakso and Voutilainen 1996). SFC permits analysis of TAGs with high boiling point or thermal sensitivity, with short analysis times and without sample derivatization. Early measurements were carried out using capillary columns (Lesellier and Tchapla 1999; Baiocchi et al. 1993; Kaplan et al. 1994; Laakso and Manninen 1997; Lesellier et al. 2000; Manninen and Laakso 1997; Manninen et al. 1995a, b), with non-polar stationary phases (principally polymethylsiloxane, phenylmethylsiloxane and octyl-methylpolysiloxane) as well as polar ones (phenyl-cyanopropylpolysiloxane, cyanopropylphenyl-methylpolysiloxane and polyethyleneglycol). The most important factor controlling separation, especially on the non-polar phases, was determined to be the acyl carbon number

(ACN). The number of double bonds (DB) influenced the separation of TAGs with the same ACN values, but in some cases TAGs with the same acyl carbon number and a different number of double bonds coeluted (Baiocchi et al. 1993; Kaplan et al. 1994). As the number of DBs determines the polarity of the TAGs, this had an opposite effect when the analytes were separated on stationary phases with opposite polarities. The research group of Pekka, Manninen et al. (1995a, b) developed several capillary SFC methods to determine TAGs in different oils. Even when increasing the column length the co-elution of several triacylglycerols could not be avoided, and the authors combined the SFC system with a triple-quadrupole mass spectrometer via a liquid-chromatography atmospheric pressure chemical ionization (LC-APCI) interphase (Manninen and Laakso 1997), using different reactant ion solvents (methanol, isopropanol, water and 0.5 % ammonium hydroxide in methanol). This method was used to identify milk fat triacylglycerols (Laakso and Manninen 1997), separated on an octyl-methylpolysiloxane column. Information on both the molecular weights and the fatty acid constituents of both saturated and unsaturated triacylglycerols was obtained. The elution of triacylglycerols having equivalent carbon numbers in a single chromatographic peak occasionally prevented correct identification of the fatty acid composition of the triacylglycerols, so the author recommended the fractionation of milk fat triacylglycerols, according to the degree of unsaturation, by argentation chromatography, prior to SFC analysis. Analysis of triacylglycerols by packed SFC has been performed by using octadecyl silica (ODS) (Lesellier and Tchapla 1999; Lesellier et al. 2000; Lee et al. 2012) or silver ion exchange (Sandra et al. 2002) columns. Lesellier (Lesellier and Tchapla 1999) studied the retention behaviour of vegetable oil triacylglycerols on ODS columns and CO_2/modifier mobile phases, as these kinds of mobile phases are suitable for separating TAGs differing in fatty acid composition. The results obtained were similar to those reported in non-aqueous reversed phase liquid chromatography or in sub-critical CO_2. With the data obtained for 30 TAGs a retention diagram was established where the relation between triglyceride series retention and their unsaturation number could be observed. The results showed a linear relationship between retention and the unsaturation number for compounds that have the same total chain or the same partition number. The relationship between retention and the triglyceride carbon number was also linear, although the nature of the modifier changed the range of retention variation. Later this group studied the possibility of using relative retention variations to identify the triacylglycerols of vegetable oils (calophyllumand peanut oils) (Lesellier et al. 2000). Separation was achieved by the use of seven ODS columns coupled in series. The compounds were identified by collecting the chromatographic peaks followed by electron impact MS. Although certain co-elutions were observed for compounds with the same total chain length, the same partition number, and the same level of unsaturation, this method was useful to predict the structures of the triacylglycerols and its accuracy was confirmed by mass spectrometric analysis. On silver ion-exchange columns (SIC) the separation of triacylglycerols is controlled by the degree and distribution of unsaturation. Sandra et al. (2002) studied the characterization of triglycerides using SIC and mass spectroscopy. TAGs were

separated according to the number of double bonds, and within the same group an additional separation was observed according to the carbon number. Two ionization modes were used: atmospheric pressure chemical ionization (APCI) and coordination ion spray (CIS) with silver ions. This last mode of ionization was introduced by Bayer et al. (1999), Rentel et al. (1999) and involved the addition, prior to nebulization, of one ion capable of forming adducts with the non-ionizable molecule. The silver ion-exchange column was prepared according to the procedure described by Christie (1988). Molecular ions $[M^{-}H^{+}]$ or $[M^{-}Ag^{+}]$ were observed in the mass spectra with both ionization modes, except for saturated triacylglycerols, for which only CIS gave intense molecular ions. APCI made it possible to determine the position where the fatty acids are esterified to the glycerol backbone; however, this could not be achieved for highly unsaturated TAGs.

Comprehensive two-dimensional packed column SFC has also been explored in the separation of complex triacylglycerol mixtures, where conventional one-dimension chromatography does not provide sufficient separation power. Hirata et al. (2003) developed a system using pure carbon dioxide as the mobile phase and UV detection. The same ODS packing material was used in both dimensions. The first column was operated under constant pressure and subcritical conditions (0–25 °C), while the second was in constant flow mode and supercritical conditions. The eluted solutes from the first dimension were trapped within a capillary trap and transferred to the second dimension using a switching valve. The system was applied to different fats and oils, permitting separation of a large number of TAGs despite the fact that saturated TAGs could not be detected due to there being no signal. More recently François et al. (2010) compared the results obtained in the separation of triacylglycerols from fish oil, using a comprehensive SFC × RP-HPLC system previously described (François and Sandra 2009), with those obtained with off-line SFC × RP-HPLC. In both cases the first dimension consisted of two serially coupled silver ion-exchange columns, whilst the mobile phase was a mixture of supercritical CO_2 and acetonitrile/IPA (6:1). In the second dimension one monolithic ODS column was used. The second dimension for off-line analysis consisted of three serially coupled C_{18} columns. The authors concluded that the complexity of the sample prevented a complete separation in the comprehensive system, mainly due to the low peak capacity in the second dimension. Better separations were obtained with the off-line approach, although the long analysis time was a disadvantage.

Phospholipids. Phospholipids are the most important class of polar lipids as they are structural components of living cell membranes and play an important role in enzyme activation, making them important in nutrition. They are commonly used as emulsifying additives, with many applications in the food, cosmetic and pharmaceutical industries. Phospholipids consist of a glycerol backbone with two adjacent carbon positions esterified with fatty acids, and generally they have an even number of carbon atoms between 14 and 22 and from 0 to 6 double bonds. The third carbon position is esterified with phosphoric acid. Common phospholipids, widely distributed in nature, are produced by further reaction of the phosphate group with an alcohol (the polar head group), such as serine, ethanolamine, choline or inositol.

Different analytical techniques have traditionally been used for phospholipid analysis, including thin layer chromatography (TLC), HPLC, GC and are adequately summarised in several reviews (Peterson and Cummings 2006; Restuccia et al. 2012). Non-chromatographic techniques include ^{31}P-NMR (MacKenzie et al. 2009). TLC has been used for qualitative separation of different classes of phospholipids but not for quantitative purposes. HPLC methods with diol, cyano or amino based columns, have been the most widely applied, but detection is the major limitation. GC cannot be employed for the analysis of intact phospholipids due to their non-volatile nature; as a consequence, GC methods depend on hydrolysis to diacylglycerols and subsequent methyl trans-esterification which generates the fatty acid methyl esters. Nowadays mass spectrometry is the selection of choice due to its high sensitivity and specificity. Although NPLC provides acceptable resolution for intact phospholipids, it is limited by the high retention and column equilibration times. Consequently, SFC could be an alternative, as it improves analysis times and resolutions. The application of SFC to the separation of phospholipids was developed initially by Lafosse et al. (1992) in the separation of phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), from soya lecithin, using isocratic conditions and evaporative light scattering detection (ELSD). This application was further examined by Eckard et al. (1998). In this case the separation of five phospholipids of differing polarity and nature was studied: 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2- dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), poly(ethylene glycolated)-1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPEPEG), 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DPPG). The study revealed a significant influence of the percentage of trifluoroacetic acid on separation. Taylor and co-workers (Yip et al. 2007) employed mass spectrometric (APCI mode) and ELSD for SFC analysis of PC, PE, PI and phosphatidylserine (PS). The study showed that PC and PE did not require additives for their elution from any of the columns, while the anionic lipids required the presence of additives, with basic and ionic additives being the most effective. The research group of Bamba is especially active in the development of lipid analysis methods using SFC-MS in APCI or ESI modes (Bamba et al. 2008; Lee et al. 2011, 2013). Although most of their work is applied to biological samples, the separation of complex lipid mixtures including phospholipids, glycolipids, neutral lipids and sphingolipids is worthy of mention. They showed that the separation of the different classes of lipids was accomplished using a cyano column, and an ODS column was more suitable for determining the fatty acid composition of the lipids (Bamba et al. 2008). Separation was by means of a gradient of modifier (methanol containing 0.1 % ammonium formate, pH 6.4). Although adequate results were obtained, the authors applied a trimethylsilyl (TMS) derivatization for methylation of the free hydroxyl groups in the phosphate moieties, in order to improve peak tailing and detection sensitivity of polar lipids.

7.4.2 Carotenoids and Fat-Soluble Vitamins

Carotenoids are one of the most widespread groups of naturally occurring fat soluble pigments and play an important role in biological processes, not only in plants but also in humans, with certain carotenoids acting as pro-vitamin A. Due to their potent antioxidant activity, they have received a great deal of attention as potential anti-cancer and anti-aging compounds, as well as prevention against cardiovascular diseases. Carotenoids can exist in *cis*/*trans* geometrical conformations, which are known to affect their biochemical activity. In fresh plant tissues, carotenoid double bonds have all-*trans* configuration but can be isomerized to the *cis* configuration, influencing their pro-vitamin A and antioxidant activity as well as their nutritional value. Chromatographic methods have been preferred for analysing these compounds, especially those based on HPLC, which have recently been reviewed (Blake 2007a, b; Rivera and Canela-Garayoa 2012). Separations have been accomplished using C_{18} or C_{30} columns, yet the main drawbacks are the relatively long analysis times and the difficulty in separating geometrical isomers. The first study dealing with SFC separation of carotenoids was published by McLaren et al. (1968). Since then, numerous papers using this technique have appeared, which indicates that SFC can be a promising alternative to traditional HPLC methods as it permits separations between the carotenoids that are more difficult to achieve, such as *cis*/*trans* isomers. Considering the sensitivity of carotenoids to temperature, low temperatures have been employed in most cases hence subcritical conditions have been applied. Lesellier et al. (1993) studied the role of 16 organic modifiers in the separation of α and β-*cis*/*trans* carotenes using sub-SFC with organic modifier, UV detection and C_{18} columns, concluding that the dielectric constant and solubility parameters were the factors that had the greatest effect on solvent elution strength; moreover, differences in the conformation of the stationary phase with the type of modifier were also observed. The modifier can alter the conformation of the stationary phase by influencing the stretching out or bending of the alkyl chains, which can decrease selectivity toward *trans*/*cis* isomers of β-carotene. The same research group further improved the separation of β-carotene *cis*/*trans* isomers (Lesellier et al. 1999). In this study they looked at different stationary phases including certain C_{18} columns: monomeric, heavily loaded and polymeric, and a polymeric triacontane bonded silica column. In all cases the separations observed were similar to those obtained in HPLC on the same stationary phases and with non-aqueous mobile phases. Separation of the four classic isomers (9-*cis*, all-*trans*, 13-*cis*, 15-*cis*) and a further six isomers was achieved in 50 min. Matsubara et al. (2009) developed an SFC-MS method to separate a mixture of β-carotene, zeaxanthin, lycopene, lutein, antheraxanthin, neoxanthin and violaxanthin. This method was applied to the analysis of green algae extracts; in this case it was difficult with only one column to identify the target compounds due to co-elution with other compounds. The problem was solved by coupling three monolithic columns. The same research group later studied the separation of β-cryptoxanthin (β-CX) and nine β-cryptoxanthin fatty acid esters

(β-CXFA) (Wada et al. 2011) and applied the method to the analysis of citrus fruits. They showed that these fruits contained a small amount of butyric, caproic, caprylic, and capric acid esters. The separation of epoxide carotenoids was also studied using a SFC-MS/MS system (Matsubara et al. 2012). These compounds are present in small amounts and are isomers of hydroxyl carotenoids, which are difficult to analyse. Abrahamsson et al. (2012) developed an SFC method for determining carotenoids in SFE extracts of microalgae (*Scenedesmus sp.*). The separation of 8 carotenoids was accomplished by coupling two columns in series, with temperature showing an influence on selectivity, where pressure did not. The best results were obtained at 32 °C. The method was validated and the limits of detection and quantification were similar to those achieved with HPLC methods.

An analysis of fat soluble vitamins by SFC was reviewed Turner et al. (Turner et al. 2001a). Open capillary columns based on polysiloxane derivatives, as well as packed microbore columns coupled to an FID detector, have been used with different degrees of success, but in recent years packed columns and UV or MS detectors have been preferred. Han et al. (2004) studied the separation of eight tocol isomers on silica, including diolmodified stationary phases, applying the proposed method to the analysis of tocols in crude palm oil. The same research group developed a SFC method to determine carotenes, vitamin E, sterols, and squalene in crude palm oil and palm fibre oil (Choo et al. 2005). Applications in carotenoid and fat soluble vitamin analysis reported by Bernal et al. (2013) are summarized in Table 7.2.

7.4.3 Vitamins

SFC is useful for vitamin analysis as an alternative to organic solvent based methods for two important reasons: oxygen absence is promoted, thus negating decomposition by oxidation; and the use of moderate temperatures also reduces the activity of thermal decomposition mechanisms. SFC fractionation of hydro-soluble vitamins (polar) has been reported with use of modifiers such as water (Pyo 2000); also the fractionation of tocopherols (vitamin E (Ibáñez et al. 1999)) and carotenoids (provitamin A (Ibáñez et al. 1998)) has been achieved by optimizing stationary phases and without co-solvent. Several methods exist for analysis and fractionation of vitamins using SFC (Turner et al. 2001a, b).

Tocochromanols are well known as components of vitamin E, and their separation may be achieved by SFC (Jiang et al. 2003; King et al. 1996; Saito et al. 1989; Upnmoor and Brunner 1989). The main subject of these publications is the investigation of the separation of different tocochromanols on different stationary phases and/or the influence of modifier, modifier concentration, temperature and pressure on the separation factor, the peak resolution and the retention factor.

Table 7.2 SFC applications in the analysis of carotenoids and fat soluble vitamins (Taylor 2009a)

Analyte	Detector	Column[a]	Mobile phase
Carotenoids (Salvador 1996)	PAD	Sun Fire C18 serially couple (to a Viridis SFC silica 2-ethylpyridine)	CO_2 + methanol
Carotenoids and epoxy carotenoids (Hoffman and Taylor 2002)	ESI-MS-QqQ	Purosphere RP-18e	CO_2 + methanol + ammonium formate
β-cryptoxanthin and β-cryptoxanthin fatty acid ester (Jentoft and Gouw 1976)	ESI-MS-QqQ	Reverse phase column, YMC carotenoid	CO_2 + methanol + ammonium formate
β-carotene, zeaxanthin, lycopene, lutein, antheraxanthin, neoxanthin and violaxanthin (Kithinji et al. 1990)	MS	Purosphere STAR RP-18e	CO_2 + methanol + ammonium formate
Carotene, vitamin E, sterols and squalene (Lesellier 1999)	UV	LiChrosorb Silica	CO_2 + ethanol
8 isomers of tocols (Gere 1983)	UV	Nucleosil 100-5OH diol	CO_2 + methyl-tert-butyl-ether
Cis/trans isomers of β-carotene (Bertoncini et al. 2001)	UV	Two hypersil ODS and Ultrabase UB 225 serially coupled	CO_2 + acetonitrile + methanol

[a]All columns 250 mm × 4.6 mm

7.4.4 Other Natural Products

Several methods use supercritical fluids to characterize natural products obtained from different plants, such as *Artemisia annua* L., *Valeriana officinalis*, *Sinapis alba* L., *Atropa belladonna* L., *Brassicacea* family, mustard, grape seed and many others (Komangerpour et al. 2002; Chester and Pinkston 2002; Kohler et al. 1997a). Compounds extracted and purified have been shown to display interesting functional properties in food and nutraceutical applications.

Two pioneering studies in this area relate to isolation of tocopherols from wheat germ and caffeine from coffee and tea (Chester and Pinkston 2002; Smith 1999). Compounds have been isolated from lemon peel using semi-preparative SFC (alcohols, aldehydes, esters and hydrocarbons) (Yamauchi and Saito 1990). The same authors also isolated tocopherols from wheat germ oil using semi-preparative SFC using two columns reaching purities of (85 and 70) % for α- and β-tocopherol, respectively (Saito and Yamauchi 1990). An example of adsorption-desorption processes is evident in the deterpenation of essential oil from citrus fruits, using on-line deodorization of plant oil extracted from seeds with supercritical CO_2 (Knez et al. 1991). This method employs a process of percolation of CO_2/extract mixture in an adsorbent bed (resins and active carbon TENAX and XAD), where adsorption capacity was described depending on pressure and temperature used. Merfort (2002) and Bos et al. (2002) included SFC in reviews of techniques for sesquiterpene and valepotriate analysis, respectively. Chromatographic resolution

of polyprenols (natural bioregulators found in various plant tissues), with chain length and geometric-isomer variations, was reported to be markedly improved with SFC over conventional HPLC separations (Bamba et al. 2001, 2003). Individual homologues containing 10–100 oligomeric units may be separated and geometric isomers isolated by SFC fractionation. The authors report that for the first time, all-*trans*polyprenols with degree of polymerization >10 were shown to exist in nature. SFC has been successfully utilized for the separation of underivatised triterpene acids (Kohler et al. 1997a, b, c; Tavares et al. 2001) and estimation of caffeine in tea (Guo 2002). Capillary-SFC has been used for analysis of panaxadiol and panaxatriol in ginseng and its preparations (Li et al. 1991), vegetable carotenoids (Schmitz et al. 1989) and pyrrolizidine alkaloids (Holzer et al. 1987).

Standardization of bacoside A3 and bacopaside II, the major triterpenoid saponins present in *Bacopamonnieri* extract, was performed using prep-SFC with photodiode-array detection, in order to improve a commercial formulation (Agrawal et al. 2006). The effect of temperature on SFC separation of the saponins was studied in detail, and Van't Hoff plots for retention and selectivity were found to be linear. The data revealed that separation of bacoside A3 was enthalpically favoured in the range of temperatures investigated; whereas entropy-controlled separation was observed for bacopaside II.

The chemical nature of davanone isolated from natural davana oil was defined, via SFC with a CO_2-based mobile phase (Coleman et al. 2007). Various analytical-scale, silica-based stationary phases were tested and a semi-preparative separation was consequently developed. The davanone fraction was almost 100 % optically pure. The results indicated that fractionation of davana oil with supercritical fluids at near room temperature had little effect on the optical integrity of the sample. Relatively large peptides (at least 40mers) containing a variety of acidic and basic residues have been eluted via SFC. Trifluoroacetic acid was used as an additive in a CO_2/ethanol mobile phase to suppress deprotonation of peptide carboxylic acid groups and to protonate peptide amino groups. The relatively simple mobile phase was compatible with mass spectrometric detection (Zheng et al. 2006). In another publication, a study was carried out to investigate the presence of coenzyme Q10 in crude palm oil and palm fibre oil by SFC with UV detection and methanol-modified CO_2 (Han et al. 2006).

There is considerable interest in the antioxidant properties of food components, but analytical methods for assessing the levels of these compounds in food are still under development. Tsao and Deng reviewed SFC along with other chromatographic techniques for analysis of phytochemical antioxidants (Tsao and Deng 2004). Resolution of the principal antioxidant compounds from rosemary using SFC (carnosic acid and carnosol) has been achieved at a relatively high pressure and temperature (Ramírez et al. 2004, 2005; Vicente et al. 2013). For preparative fractionation of a supercritical fluid extract of rosemary, a custom designed column was developed to allow fraction collection (Ramírez et al. 2007). The column was prepared using a self-packing method, including use of supercritical CO_2, applied to commercial silica particles commonly used in GC. The new procedure provided columns with reasonable efficiencies and high stability at high pressure. Separation

power was tested for isolating fractions with high antioxidant and/or antimicrobial activity.

Triterpenoid compounds are typically difficult to analyse by GC or HPLC due to their low volatility and low UV absorbance. However, Lesellier et al. employed packed SFC and ELSD for studying the separation of eight such triterpenoids on various stationary phases (Lesellier et al. 2012). The conclusion was drawn that an ethyl-pyridine bonded column was best suited for class separation of mono-ol, diol and acidic triterpenoids. Nevertheless, the best separation of the compounds studied was obtained with a reverse phase column. The order of retention on this column was mono-ol < acidic < diol triterpenoids, and within each of these groups the retention order was oleane type < ursane type < lupine. The proposed method was applied to the analysis of apple pommace extract. Polyphenols from SFE grape extracts were determined by Komangerpour et al. using SFC (Komangerpour et al. 2002). Different stationary phases (diol, cyanopropyl and silica) were studied and the best results were obtained by coupling two diol columns, whereby eight compounds (2-phenyl-ethanol, vanillin, ferulic acid, protocatechoicacid, caffeic acid, gallic acid, catechin and epicatechin) were efficiently separated. Separation of polymethoxylated flavones (tangeretin, heptamethoxyflavone, nobiletin, tetra-O-methylscutellarein, hexamethoxyflavone and sinensetin) (Dugo et al. 1996) has been studied, with a resultant method applied to the quantitative analysis of different samples of mandarin and orange oils. Similar results were obtained to those achieved using HPLC, with the advantage of a reduction in analysis time by a factor of 4. Carbohydrates have also been analysed using SFC, as was reviewed by Lafosse et al. (1996); highlighting the separation of eight monosaccharides and polyols by means of a water modified mobile phase (up to 9 %) (Salvador et al. 1997). More recently, Lefler and Chen separated a mixture of caffeine, fructose, glucose, sucrose and neohesperidine dihydrochalcone, an artificial sweetener, utilizing evaporative light scattering detection (Lefler and Chen 2008). The same method may be used to determine the presence of sucralose in sports drinks.

SFC has also been applied in the area of food adulterants. Another interesting application of SFC is the separation of sinalbin (4-hydroxybenzylglucosinolate) degradation products (Buskov et al. 2000). The separation of five compounds (4-hydroxybenzyl isothiocyanate, sinapic acid, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol and 4-hydroxybenzylascorbigen) was performed and the method was applied to determining these compounds in different mustards and mustard powders.

7.4.5 Preparative Separations

Preparative-SFC (prep-SFC) is a potentially valuable commercial tool, and in some cases complementary to prep-HPLC, for isolating different compounds of a mixture, as in the case of producing functional food ingredients; it is frequently used for

the fractionation and purification of extracts obtained with supercritical fluid extraction (SFE). Semi-preparative separation of crude palm oil components has been studied (Choo et al. 1996); the four fractions consisted of free fatty acids, thentocopherols, tocotrienols and diglycerides, then isomeric triglycerides, and then carotenes and triglycerides, respectively. Phytosterols have also been isolated in this way, a notable example of which is by Taylor and King (2002), who combined prep-SFE with SFC to obtain fractions enriched in free sterols and ferulate-phytosterol esters (FPEs) from corn bran. The corn bran oil was first extracted with neat CO_2, in high yield. The subsequent online SFC fractionation process was accomplished in three steps: the first was performed with neat $CO_2$with most of the TAGs and phytosterol fatty acyl esters removed at this stage. The second step, facilitated by addition of ethanol as a solvent modifier, was designed to maximize FPE enrichment, and in the third step with increased ethanol content, any remaining compounds were eluted from the column to prevent extract carryover to subsequent runs. With these conditions a fraction exhibiting enrichment by more than fourfold in free sterols and tenfold in FPE was obtained.

A method for efficient and relatively large-scale isolation of four polymethoxyflavones (PMFs) from sweet orange (*Citrussinensis*) peel by employing SFC was reported (Li et al. 2007). PMFs represent a class of compounds with important biological activities (anti-inflammatory, anti-carcinogenic and anti-atherogenic). The flavones present were nobiletin, tangeretin, 3,5,6,7,8,3′, 4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone. SFC technology had a dramatic advantage over other separation methods and was found to be well suited for providing large amounts of PMFs from orange peel extract. A mixture of CO_2 and different percentages of methanol containing 0.25 % diethylamine was employed as the mobile phase. In the case of tangeretin and 3,5,6,7,8,3′, 4′-heptamethoxyflavone, one purification cycle took less than 7 min; thus 25 purification cycles could be performed in less than 3 h, in which more than 700 mg of product was obtained. In the case of nobiletin and 5,6,7,4′-tetramethoxyflavone, separation was achieved in under 8 min, which was purported to display a clear advantage over preparative HPLC procedures.

A supercritical fluid extract of rosemary has been fractionated using a prep-SFC system (Ramírez et al. 2006). The selective isolation of the compounds responsible for both antioxidant and antimicrobial activities was of interest. Two cyclones were employed to collect the fractions which were subsequently characterized by HPLC-DAD (photodiode array detection), GC, and *in vitro* antioxidant and antimicrobial assays. By careful selection of separation conditions two different fractions were obtained, one enriched in antioxidant and antimicrobial compounds collected in one cyclone with no residual rosemary aroma and a second fraction (in the other cyclone) that contained the essential oil. Semi-preparative SFC was also employed by García-Risco et al. (2011) for fractionation of SFE thymus extracts resulting in a fraction containing 97 % of the thymol content.

7.5 Trends and Opportunities

SFC applications in the area of food analysis have traditionally been focused on lipid compounds, due to the high solubility of these analytes in supercritical CO_2. However, this trend is changing. Several studies have shown the capacity of SFC for analysis of more polar compounds, such as amino acids or carbohydrates, by using mobile phases modified with a certain proportion of water. Moreover, the possibility of using columns with different functionality and employing the same mobile phase, in conjunction with the feasibility of serially coupling columns, has made it possible to enlarge the range of compounds that can be separated in the same run. The analysis of complex mixtures has been accomplished by using 2D SFC or coupling SFC to RP-HPLC, but increasingly the SFC-MS combination is now being chosen, providing very good levels of sensitivity. On a preparative scale, SFC is a suitable alternative to liquid chromatography, with sound results in the isolation of food components with a high bioactive value, such as fatty acids or tocopherols.

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Chapter 8
Direct and Indirect Applications of Sub- and Supercritical Water in Food-Related Analysis

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8.1 Water as the Greenest and the Most Tunable Solvent

The growing emphasis on sustainable development and on the use of environment- and resource-conscious technologies results in increasing use of green solvents, with water taking a prominent position. In addition to being the greenest of solvents, water is also the most tunable solvent as regards the variability of the solvent properties that can result from the changes in the operating temperature (T) and pressure (P) (Shaw et al. 1991; Savage 1999; Palmer et al. 2004; Weingärtner and Franck 2005).

The physical properties relevant to the solvent power of water include the density ρ, the solubility parameter δ (=the square root of the cohesive energy density c) (Prausnitz et al. 1999), the internal pressure P_{int}, the relative permittivity ε_r, and the ion product K_w. At a particular temperature and pressure, numerical values of the properties can readily be calculated using, e.g., the Wagner–Pruss equation of state (Wagner and Pruss 2002; Wagner and Overhoff 2006) for ρ, c, δ and P_{int} and literature correlations for ε_r (Uematsu and Franck 1980; Fernández et al. 1997) and K_w (Marshall and Franck 1981; Bandura and Lvov 2006). Figure 8.1 shows the calculated properties as functions of T and P; in Fig. 8.1e, $pK_w = -\log_{10} K_w$, with K_w expressed in $(\text{mol.kg}^{-1})^2$. Note that, unlike the solubility parameter surface in Fig. 8.1b, the internal pressure surface in Fig. 8.1c does not resemble the density surface in Fig. 8.1a. The plots provide an expressive illustration of tunability of the water solvent properties through adjustments of the operating T and P. To put the figures into a broader perspective, Table 8.1 lists some of the above-mentioned properties for a selection of common organic solvents and water at 25 °C and 0.1 MPa. The data shown in Table 8.1 were calculated from the equations

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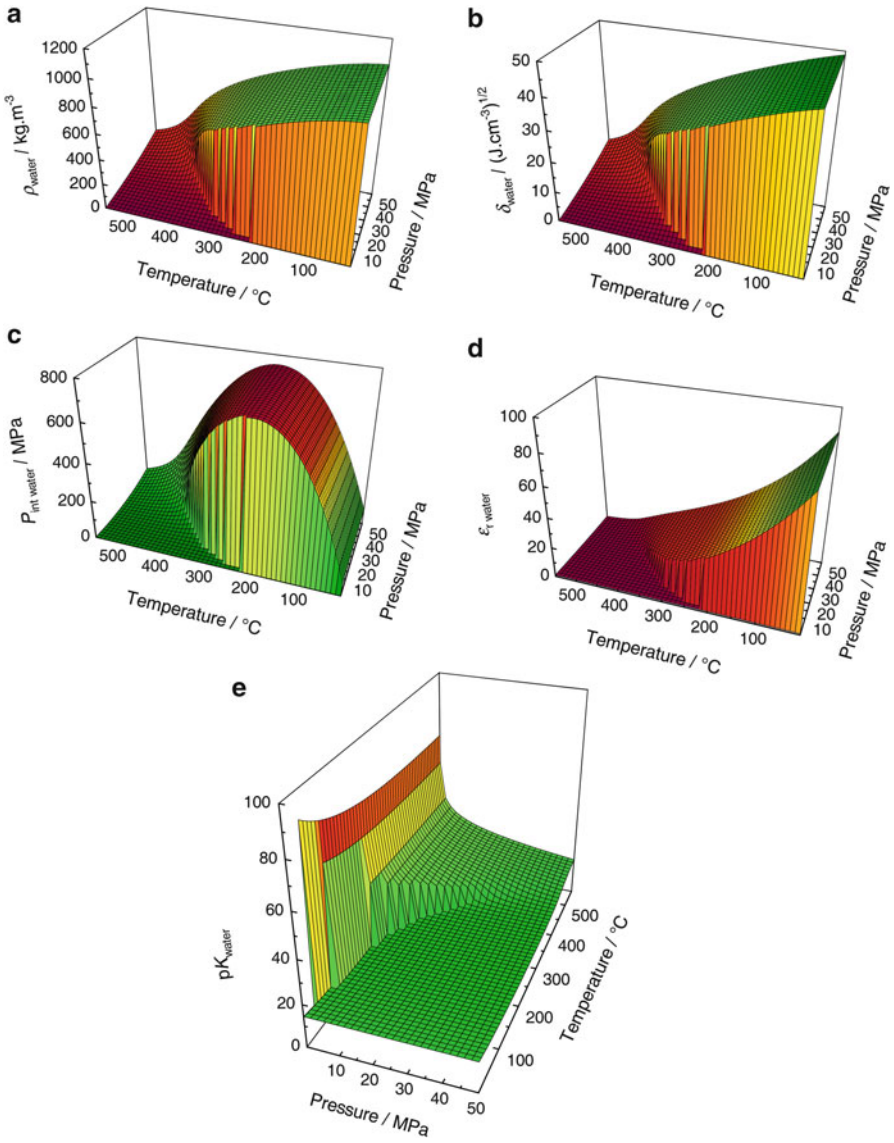

Fig. 8.1 Solvent properties of water as functions of temperature and pressure: (**a**) density; (**b**) solubility parameter; (**c**) internal pressure; (**d**) relative permittivity; (**e**) ion product ($pK_w = -\log_{10} K_w$, K_w in [mol kg^{-1}]^2)

of state of the individual solvents (Wagner and Overhoff 2006) except for the ε_r values that came from a literature source (Lide 2004). It turns out that, at high T and P, the δ and ε_r values for water may even become lower than the respective data for *n*-hexane at 25 °C and 0.1 MPa (compare Figs. 8.1b, d with Table 8.1).

Table 8.1 Properties of Selected Solvents at 25 °C and 0.1 MPa

Solvent	ρ/g cm^{-3}	c/J cm^{-3}	δ/(J cm^{-3})$^{1/2}$	P_{int}/MPa	ε_r	$n = P_{int}/c$
Cyclohexane	0.7739	281.2	16.77	321.6	2.02	1.144
n-Pentane	0.6208	208.7	14.45	226.2	1.83	1.084
n-Hexane	0.6548	221.7	14.89	236.8	1.88	1.068
Toluene	0.8622	333.1	18.25	349.9	2.37	1.050
Benzene	0.8727	351.1	18.74	362.0	2.27	1.031
Acetone	0.7847	390.3	19.76	326.6	20.49	0.837
Ethanol	0.7855	682.5	26.12	278.2	24.85	0.408
Methanol	0.7863	874.3	29.57	281.4	32.61	0.322
Water	0.9970	2,299	47.95	169.4	78.45	0.074

Another illustration of the variable solvent properties of water may be based on comparison of the properties c and P_{int}. The cohesive energy density is defined by

$$c = \Delta u_c / v \tag{8.1}$$

where v is the molar volume of the fluid at the particular T and P and Δu_c is the fluid's cohesive energy, i.e., the energy required for isothermal vaporization of 1 mol of the fluid into an ideal gas at zero pressure. In turn, the internal pressure of a fluid is a differential quotient measuring the response of the fluid's internal energy to an infinitesimal change in volume at a constant temperature. P_{int} is defined by

$$P_{int} = (\partial u / \partial v)_T \tag{8.2}$$

and it is related to measurable properties by

$$P_{int} = T(\partial P / \partial T)_v - P \tag{8.3}$$

The cohesive energy Δu_c and, consequently, c reflect the total energy needed to break all intermolecular interactions in the fluid. On the contrary, it has been suggested (Wiehe and Bagley 1967) that P_{int} only reflects the breakage of non-specific intermolecular interactions as hydrogen bonds have been assumed not to be disturbed by an infinitesimal change in the fluid's volume (Dack 1975). Therefore, the ratio of the internal pressure to the cohesive energy density (n) may be viewed (Reichardt 2004) as a macroscopic-property-based reflection of the relative importance of specific interactions (H-bonding) in the overall solvent–solvent intermolecular interactions. As illustrated by Table 8.1, the more "polar" is the solvent, the lower is the pertinent value of n. Figure 8.2 shows that, depending on the T and P, the value of n in water varies in rather broad range reflecting the widely varying importance of water–water hydrogen bonds in the T and P region concerned. With the rising T and P, the relative importance of hydrogen bonding in water decreases and the value of n rises becoming similar to the n values of nonpolar organic solvents at 25 °C and 0.1 MPa (see the last column of Table 8.1).

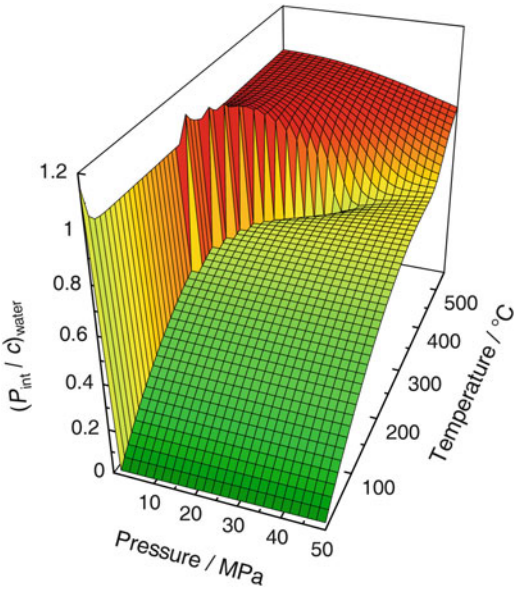

Fig. 8.2 Ratio of the internal pressure of water to the cohesive energy density of water as a function of temperature and pressure

Therefore, the position of water among other solvents is unique in that there is no other single-component solvent offering a comparable range of tunability of the solvent properties through changes in the operating temperature and pressure. This is the basis for diverse applications of high-temperature water as a solvent or reaction medium (Brunner 2001; Savage 2009; Loppinet-Serani et al. 2010; Möller et al. 2011; Toor et al. 2011; Carr et al. 2011; Machida et al. 2011).

8.2 Subcritical Water as an Extraction Agent: *Pros* and *Cons*

Wide-range tunability of the solvent properties of water through the changes in operating temperature and pressure leads to some applications that may apparently seem to contradict the conventional chemical "common sense". One of the prerequisites for the use of pressurized hot water (PHW) as an extraction agent has been a sufficient solubility of the target compounds in PHW. Therefore, this section opens with a brief discussion of solubility of organic compounds in PHW.

The decrease in polarity of water with the rising temperature contributes to the concomitant increase in the aqueous solubilities of low-polarity organic nonelectrolytes. Consequently, there have been numerous measurements of the aqueous solubilities of organics at high temperatures (Dohrn et al. 2010; Fonseca et al. 2011; Škerget et al. 2011). Although some of the studies have covered the solutes that are liquid at the measurement temperatures (Yang et al. 1997; Chandler et al. 1998; Miller and Hawthorne 2000a, b), a significant part of these efforts have dealt with

the solubilities of organic nonelectrolyte solids. The main reason is probably that the solid phase at equilibrium with the aqueous solution remains a pure solid solute as opposed to most situations when the solute is a liquid at the measurement temperature. As a result, the experimental arrangement and procedure for the solid solubility measurements are simpler as compared to those with the liquid solutes. Moreover, the solid solubility measurements lend themselves to an easier thermodynamic analysis, again because the solid phase can be considered a pure solute.

The early studies of aqueous solubilities at high temperatures involved nonpolar solutes such as heavy alkanes (Sanders 1986) and aromatic hydrocarbons (Rössling and Franck 1983). Later on, the measurements were extended to cover more polycyclic aromatic hydrocarbons (PAHs) (Miller and Hawthorne 1998; Miller et al. 1998; Andersson et al. 2005; Karásek et al. 2006a, b) and other classes of solutes including aromatic hydrocarbons (Karásek et al. 2008a), diamondoids (Karásek et al. 2008b), aromatic heterocycles (Miller et al. 1998; Karásek et al. 2007, 2009), benzoic and salicylic acids (Kayan et al. 2010), ferrocene (Karásek et al. 2010a), terephthalic acid (Takebayashi et al. 2012) and organic electronic materials (Karásek et al. 2013a). As an example, Fig. 8.3 illustrates the increasing solubilities of a selection of solutes with the rising temperature whereas Fig. 8.4 shows the concomitant decrease in the respective solute activity coefficients (Karásek et al. 2009).

However, although expanding the solubility data base and serving to test thermodynamic models of aqueous systems at elevated temperatures, the above solutes bore no direct relevance to the topic of food processing. Recently, however, solubility measurements have appeared on the solutes relevant to food processing and analysis including quercetin and quercetin dihydrate (Srinivas et al. 2010a), carbohydrates (Zhang et al. 2010; Saldaña et al. 2012), and gallic acid, catechin and protocatechuic acid (Srinivas et al. 2010b). High-temperature aqueous solubility

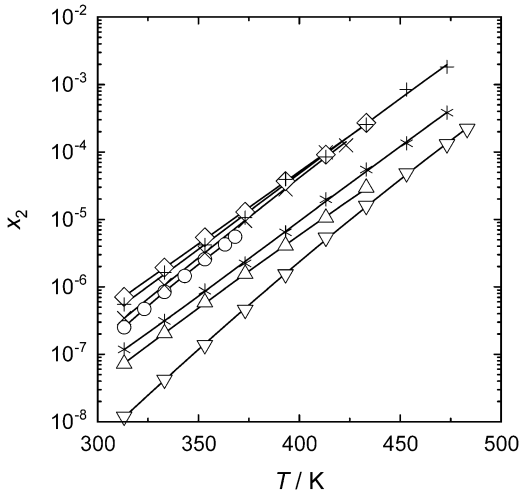

Fig. 8.3 Aqueous solubilities of oxygenated aromatic solutes: ○, xanthene; ×, anthrone; ◇, xanthone; *, thioxanthone; Δ, 9,10-anthraquinone; +, 9,10-phenanthrenequinone. Anthracene (Karásek et al. 2006a) ▽ is shown for reference. The lines serve just to guide the eye. Reprinted with permission from Karásek et al. (2009). Copyright 2009 American Chemical Society

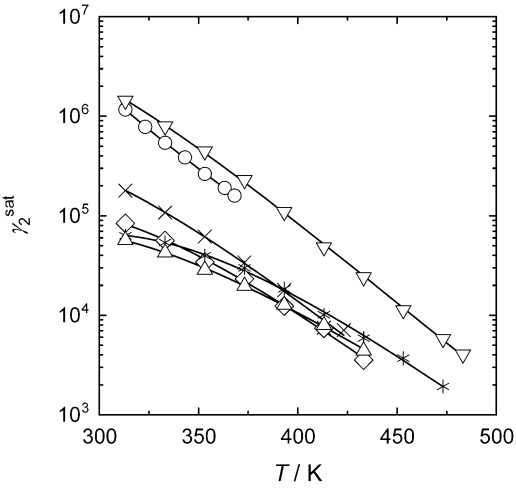

Fig. 8.4 Activity coefficients of oxygenated aromatic solutes in saturated aqueous solutions: ○, xanthene; ×, anthrone; ◇, xanthone; *, thioxanthone; Δ, 9,10-anthraquinone. Anthracene (Karásek et al. 2006b) ▽ is shown for reference. The lines serve just to guide the eye. Reprinted with permission from Karásek et al. (2009). Copyright 2009 American Chemical Society

data on selected pharmaceuticals have also been available including griseofulvin (Carr et al. 2010a), naproxen (Carr et al. 2010b) and budesonide (Carr et al. 2010c).

Given the application importance of the aqueous solutions of organics, considerable effort has been spent on the development of correlations and/or predictive models of the solubilities of organic solutes in PHW. The specific feature of the models for this purpose is that they have to work satisfactorily over very wide intervals of temperature (~200 °C or more). The approaches taken ranged from the use of general models to specific correlations aimed at the description of the aqueous solubilities for a specific class of solutes. A portion of the general models have been based on various thermodynamic approaches used before to describe the composition dependence of the activity coefficients in the liquid mixtures such as cubic-plus-association equation of state (Oliveira et al. 2009), UNIQUAC, NRTL, Wilson, van Laar and regular solution equations (Alvarez and Saldaña 2011), COSMO-SAC theory (Saldaña et al. 2012), regular solution theory-based approach (Fornari et al. 2011), corresponding-states theory-based treatment (del Valle et al. 2011), Peng–Robinson (PR) equation of state (Teoh et al. 2013) or NRTL–PR combination (Escandell et al. 2014).

Another portion of the general models have made use of the group additivity concept, i.e., the presumption that the logarithm of the solute activity coefficient can be built up as a sum of contributions from the individual groups constituting the solute molecule. Fornari et al. (Fornari et al. 2008, 2011) explored the capabilities of three different versions of the well-known UNIFAC model (Fredenslund et al. 1975, 1977; Gmehling et al. 1993; Gmehling 2009) to predict the solubilities of diverse solid solutes in PHW within a wide range of temperature. The results of application of the UNIFAC-with-association model indicate that the gradual breakdown of association between water molecules with the rising temperature contributes to the increased solubilities of hydrophobic organic compounds. A modified

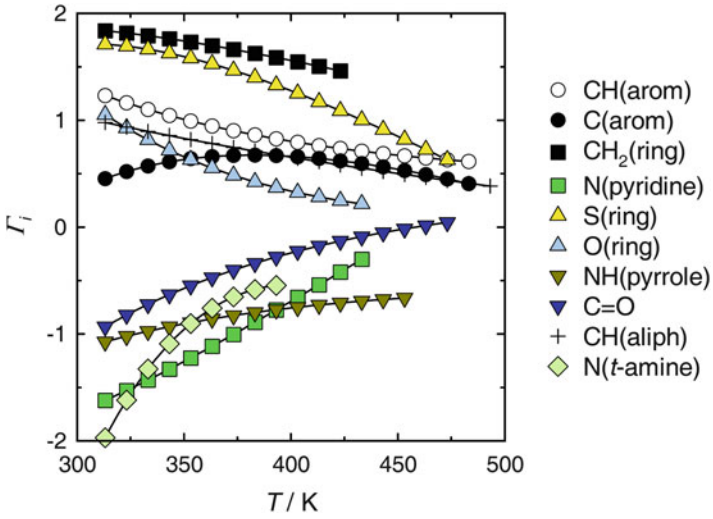

Fig. 8.5 Group contributions Γ_i to the logarithm of the solute activity coefficient in the saturated aqueous solution. Adapted with permission from Karásek et al. (2013a). Copyright 2013 Elsevier Science

UNIFAC model has also been used by Carr et al. to describe the aqueous solubilities of selected pharmaceuticals (Carr et al. 2010a, b, c), and the UNIFAC-with-association model has been employed by Zhang et al. (Zhang et al. 2010) to correlate the aqueous solubilities of carbohydrates (xylose, glucose and maltose monohydrate).

A simpler version of the group additivity model has been developed by Karásek et al. (Karásek et al. 2008c, 2010b) with a specific aim to describe the aqueous solubilities over a wide range of temperature. The above model may be viewed as "condensed experimental data" because it does not involve any theoretical presumptions except for the group additivity concept in the solute activity coefficients. Figure 8.5 (Karásek et al. 2013a) shows the temperature-dependent contributions of the individual groups or atoms as regressed from the solute activity coefficients obtained from the experimental solubility data. Although the model is far from rigorous because the group contributions do not refer to a constant composition of the aqueous solution, it provides another illustration of the changing nature of water as a solvent with the rising temperature. It appears that, as the temperature increases, the hydrophobic atoms/groups ($\Gamma_i > 0$) become less hydrophobic while the hydrophilic atoms/groups ($\Gamma_i < 0$) become less hydrophilic, with the respective group contributions approaching the Raoult-law ideality limit ($\Gamma_i = 0$) either from above or from below. Figure 8.5 thus provides additional indication of the improving ability of water to solvate organic nonelectrolyte solutes with the increasing temperature.

The (semi)empirical models developed to correlate the aqueous solubilities of a specific class of solutes across wide intervals of temperature were largely focused on aromatic hydrocarbons (Miller et al. 1998; Karásek et al. 2006b; Mathis et al. 2004).

Because of the tunable solvent characteristics of water discussed above, PHW has become increasingly frequented as the extraction solvent for a wide range of organic compounds from diverse materials and matrices (Ramos et al. 2002; Smith 2006; Kronholm et al. 2007; Nerín et al. 2009; Teo et al. 2010; Sun et al. 2012). Although the early applications of pressurized hot water extraction (PHWE) were concentrated on low-to-moderate polarity compounds and matrices relevant to environmental protection (Hawthorne et al. 1994; van Bavel et al. 1999), applications related to food processing and/or analysis were soon to follow (Basile et al. 1998; Pawlowski and Poole 1998; Rovio et al. 1999); obviously, no other solvent can match water as regards the safety in and compatibility with food-related applications, and PHWE has become established among the diverse methods used to recover valuable substances from plant materials (Wijngaard et al. 2012; Gil-Chávez et al. 2013; Bucar et al. 2013). Consequently, there have been a very large number of studies on PHWE of food-related samples including extractions of high-value substances used as health-promoting additives in functional foods, and this field has frequently been reviewed (Smith 2002; Carabias-Martínez et al. 2005; Mendiola et al. 2007; Mustafa and Turner 2011).

Owing partly to its variable solvating abilities, water is not only a tunable solvent but also a versatile reaction medium and a reagent. The boundaries among these individual roles of water are somewhat blurred as they depend on the operating conditions (temperature, pressure, the presence of oxidants) as well as on the character of the organic portion of the system. Examples of applications of water as a reaction medium or reactant may include, e.g., supercritical water oxidation (Bermejo and Cocero 2006; Marrone 2013; Vadillo et al. 2013), gasification of carbohydrates in supercritical water (Kruse 2008; Pavlovič et al. 2013) or hydrothermal liquefaction of biomass to fuels (Kruse et al. 2013) although high-temperature water can also serve as a reaction medium for synthesis processes (Kruse and Dinjus 2007; Hayashi and Hakuta 2010; Adschiri et al. 2011). Degradation reactions in high-temperature water can broadly be divided into hydrolytic (including hydrothermal) processes (Brunner 2009a) and oxidative processes (Brunner 2009b). From the viewpoint of application of water as an extraction solvent, it is very important to note that even some organic substances conventionally labeled as stable can undergo chemical alterations in water at considerably milder temperatures than those encountered in supercritical water. For example, even polycyclic aromatic hydrocarbons were repeatedly shown to undergo chemical transformations in contact with near-critical water (Teoh et al. 2013; Andersson et al. 2003; Yang and Hildebrand 2006). Expectedly, therefore, with the temperature- and/or hydrolysis-sensitive substances relevant to food-related applications, the question of their stability during PHWE becomes still more pressing. Actual or possible decomposition of the target compounds during PHWE has been reported in a large number of studies concerning, e.g., oxygenated and non-oxygenated terpenoids from savory and peppermint (Kubátová et al. 2001), floral oil components

from *Rosa canina* (Özel and Clifford 2004), monoterpenoid hydrocarbons and oxygenated compounds from basil and oregano leaves (Yang et al. 2007), quercetin and quercetin glycosides from onions or onion waste (Turner et al. 2006; Lindahl et al. 2013), naringenin and other flavonoids from aspen knotwood (Hartonen et al. 2007), stevioside from *Stevia rebaudiana* leaves (Pól et al. 2007), gastrodin and vanillyl alcohol from tubers of *Gastrodia elata* orchid (Teo et al. 2008), betulin and antioxidants from birch bark (Co et al. 2009) or cyanidin and delphinidin glycosides from red onion (Petersson et al. 2010).

Possible chemical alterations that may accompany PHWE are not limited just to degradations, and some of them may actually increase the application value of the resultant extracts, notably their antioxidant capacities. There have been several reports of neoformation of antioxidants during or after PHWE of plant materials (Plaza et al. 2010a, b, 2013). The neoformation of antioxidants may occur through the Maillard reaction involving the condensation of the carbonyl group of reducing sugars, aldehydes or ketones and the primary amine group of amino acids, peptides or proteins. The Maillard reaction products possess various biological properties including antioxidant capacity (Silván et al. 2006), and they have been shown to contribute to the overall antioxidant capacity of thermally processed foods (Morales and Babbel 2002). Caramelization (sugar break-down) is another process that can occur during PHWE simultaneously with the Maillard reaction (Plaza et al. 2010a) and that may also lead to formation of products with antioxidant properties (Tsai et al. 2009). Neoformation of antioxidant compounds in PHWE has been shown to occur in glycation model systems (Plaza et al. 2010a), in the extraction of microalgae, algae and rosemary, thyme and verbena plant leaves (Plaza et al. 2010b), and also in the extraction of apple byproducts (Plaza et al. 2013). Another compound to be explicitly mentioned here in connection to chemical changes during PHWE is 5-hydroxymethylfurfural, a highly reactive, intermediate compound that can be formed by the Maillard and caramelization reactions. 5-Hydroxymethylfurfural has been commonly found in thermally processed, carbohydrate-rich foods and has also been shown to be formed during PHWE of olive leaves (Herrero et al. 2012) and apple byproducts (Plaza et al. 2013).

In turn, if the target compounds are sufficiently stable, the operating temperature may be used to adjust the selectivity of PHWE such as in the extraction of antioxidant compounds from rosemary plants (Ibañez et al. 2003), in the extraction of prenylflavonoids from female hop plants (Gil-Ramírez et al. 2012) or in a large study involving extraction of several flavonols, flavones and flavanones from samples of various cultivated plants (Ko et al. 2014).

More often than not, however, temperature- and/or hydrolysis-sensitive substances (notably glycosides) have been shown to degrade during PHWE of plant materials. To give an example, Šťavíková et al. (Šťavíková et al. 2011) used electron paramagnetic resonance (EPR) spectroscopy to evaluate the antioxidant activities of the aqueous extracts of red grape skins from PHWE under relatively mild conditions (temperature up to 120 °C). Grape skins, usually a waste after winemaking, are a prospective and prolific source of valuable substances because only about 30–40 % of polyphenolic antioxidants contained in the skins are

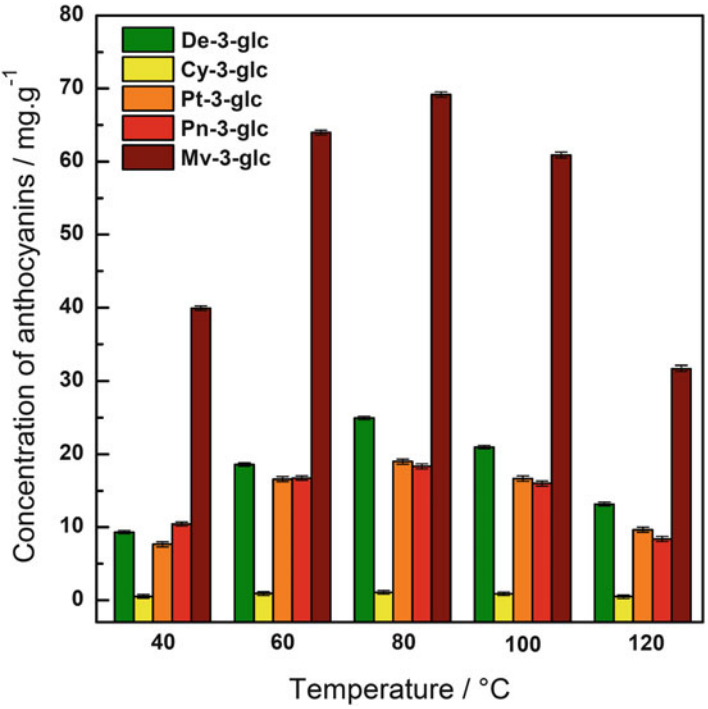

Fig. 8.6 Concentrations of anthocyanidin-3-O-glycosides in aqueous extracts of St. Laurent wine variety grape skins (extraction temperatures 40–120 °C). Adapted with permission from Šťavíková et al. (2011). Copyright 2011 Elsevier Science

transferred to wine during the winemaking process (van Balen 1984; Luque-Rodríguez et al. 2007). The PHWE–EPR study (Šťavíková et al. 2011) served to determine the total antioxidant activity of the extracts. Concentrations of five selected anthocyanidins in the extracts were determined by HPLC. Figure 8.6 shows the concentrations of the five antioxidants in the aqueous extracts of the dry skins of St. Laurent variety red grapes in dependence of the extraction temperature. The decreased concentrations at the two highest temperatures obviously result from degradation of the anthocyanins although no attempt has been made to identify the degradation mechanism. In fact, a similar course of the concentrations of the same anthocyanins with temperature has been observed when the same grape skins have been used in pressurized solvent extraction with ethanol in the same temperature range (Polovka et al. 2010).

Together with the previous reports (Kubátová et al. 2001; Özel and Clifford 2004; Yang et al. 2007; Turner et al. 2006; Lindahl et al. 2013; Hartonen et al. 2007; Pól et al. 2007; Teo et al. 2008; Co et al. 2009; Petersson et al. 2010), the above example indicates that there seems to be a limit on practicability of PHWE in food-related applications. The limit has been imposed by stability of the target substances under the operating conditions of PHWE (notably the operating temperature).

8.3 Benefits of Supercritical Water–Silica Interactions for Analytical Separation Devices

It was illustrated in the preceding section that pressurized hot (subcritical) water is not generally compatible with the solutes of biological and/or food-processing relevance because of the constraints imposed by chemical stability of these solutes in high-temperature water. Therefore, direct applications of PHW as an extraction agent or a chromatographic carrier fluid for these solutes are more or less limited. Indirectly, however, sub- and even supercritical water can still be highly useful in analytical separation methods including those employed in food-related analyses. This is because of the fact that very hot water dissolves silica and that, consequently, sub- and supercritical water can be employed to modify the inner surface and/or internal diameter of fused silica capillaries to be used in analytical separation methods. The general goal of such modifications is to improve the separation efficiency (or even enable the separation of the particular analytes at all). The ensuing improvements in both qualitative and quantitative analyses then translate into the diverse fields where the needs for the analyses have come from, including the food science, food processing and food quality control.

The discussion of the aqueous solubility of fused silica at high temperature and pressure is perhaps best started with a brief excursion to geochemistry. In that field, the interactions between silicon dioxide and water are highly important as hydrothermal solutions of SiO_2 play an essential role in crystallization and deposition of quartz and other siliceous minerals (Liebscher 2010). Since quartz is the most important and the most abundant crystalline form of SiO_2, there have been many experimental measurements of the quartz solubility in water at high temperature and pressure (Kennedy 1950; Anderson and Burnham 1965; Walther and Orville 1983; Newton and Manning 2009; Hemley et al. 1980), and correlations are available to model the aqueous solubility of quartz as a function of temperature and density of water (Walther and Helgeson 1977; Manning 1994; Dolejš and Manning 2010). As compared to the quartz–water interactions, the amount of information on the interactions between water and amorphous silica at high temperature and pressure is relatively scarce (Fournier and Rowe 1977; Chen and Marshall 1982; Fournier and Marshall 1983) because of much lower demand. Since fused silica is one of the forms of amorphous silica, it may be expected that, at a particular temperature and pressure, fused silica should be more soluble in water as compared to quartz although it is not readily obvious just how much more soluble fused silica is in comparison to quartz.

However, for the emerging applications of sub- and supercritical water as an agent to modify fused silica capillaries it would be useful to have a quantitative tool available to predict the aqueous solubility of fused silica. For this purpose, therefore, we have developed a simple correlation requiring the temperature, pressure and density of pure water as the input data (Karásek et al. 2013b). The correlation combines the literature models of the aqueous solubility of quartz (Manning 1994; Dolejš and Manning 2010) with the thermophysical properties of fused silica and

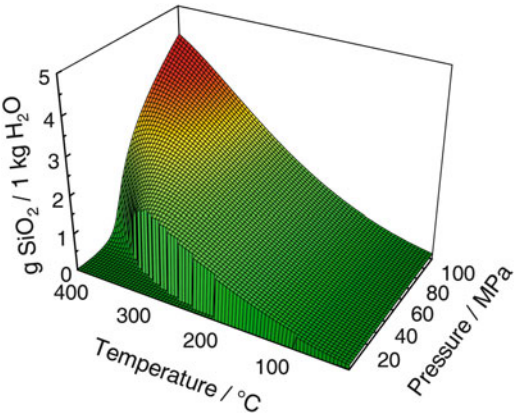

Fig. 8.7 Aqueous solubility of fused silica (mass of SiO_2 per 1 kg of water) as a function of temperature and pressure. Adapted with permission from Karásek et al. (2013b). Copyright 2013 Elsevier Science

quartz (Richet et al. 1982) and with an empirical description of the activity coefficients of SiO_2 in the aqueous solution. Figure 8.7 illustrates the estimated aqueous solubility of fused silica as a function of temperature and pressure suggesting that sub- and supercritical water should indeed be capable of serving as the surface treatment agent for fused silica capillaries.

Karásek et al. (Karásek et al. 2013c) have described the design and some applications of a multi-purpose laboratory reactor/extractor to work with supercritical water including the ability to treat any length of a fused silica capillary. Figure 8.8 shows a schematic diagram (a) and a photograph (b) of the apparatus. The instrument can be operated in three different modes, namely, static mode, dynamic mode, and semi-dynamic mode.

The static mode involves treating of the inner surface of the capillary with stagnant (supercritical) water inside at a fixed temperature and pressure, i.e., without a pressure drop along the capillary length. However, the experiments with a 100 μm i.d. capillary have shown that, in a wide range of operating temperature and pressure, the small amount of water inside the capillary quickly becomes saturated with the dissolved SiO_2 and the resultant effect on the capillary surface is barely noticeable.

In turn, the dynamic mode involves treating the inner surface with supercritical water flowing through the capillary. To support the flow, there must be a pressure gradient along the capillary in the dynamic mode, and it is also accompanied by gradients in the solvent properties of water that depend on T and P (see Sect. 8.1). As there is a steady inflow of fresh, SiO_2-free supercritical water into the capillary, the effects on the inner surface are much more pronounced as compared to those in the static mode, and they involve the surface roughening as well as alterations in the internal diameter of the capillary. The action of flowing supercritical water can lead to significant erosion of the capillary wall leaving just a very thin shell of silica in place of the original capillary wall, as illustrated in Fig. 8.9. The internal diameter profiles shown in Fig. 8.9 present the outcome of a delicate interplay between the local concentration of SiO_2 in the aqueous solution, the equilibrium solubility of

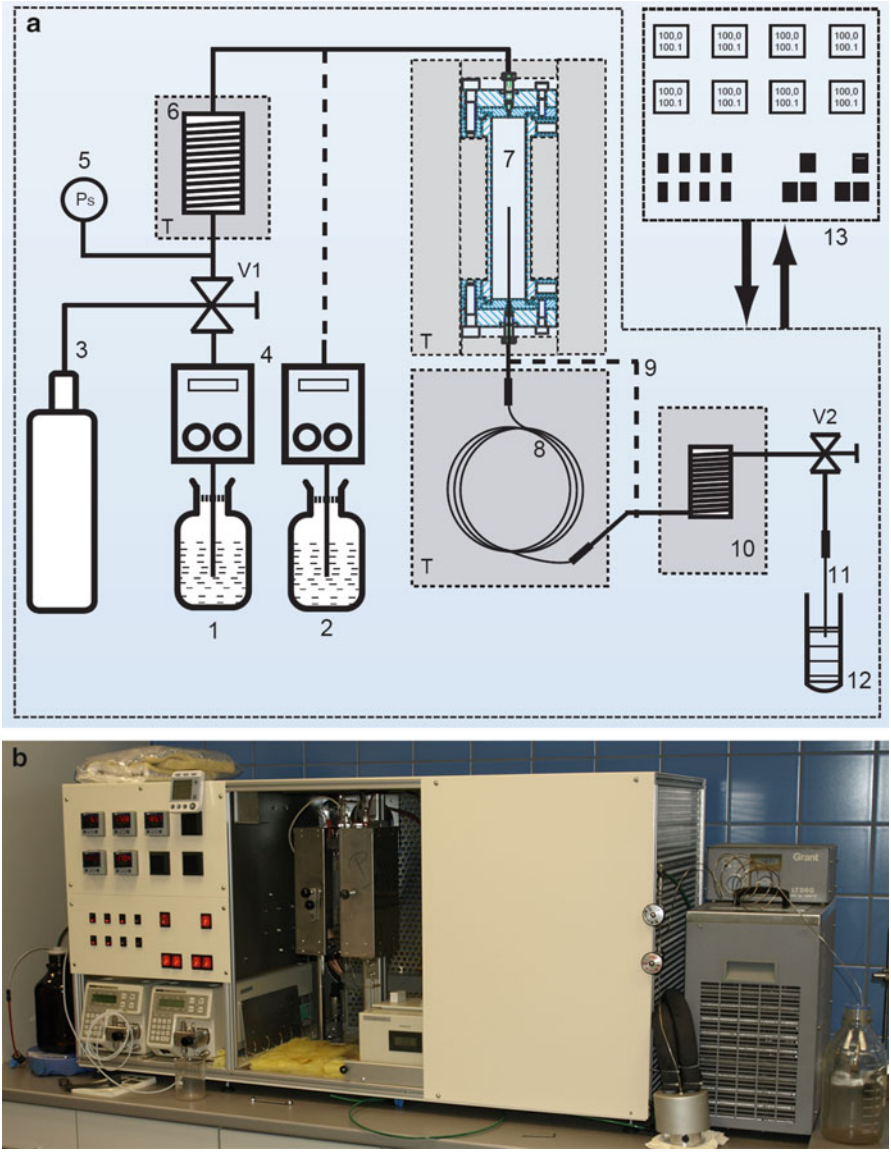

Fig. 8.8 Schematic diagram (**a**) and photograph (**b**) of a multi-purpose apparatus for applications of supercritical water. Reprinted with permission from Karásek et al. (2013c). Copyright 2013 American Chemical Society

SiO_2 at the local temperature and pressure, and the gradients of the solvent properties of water along the capillary. In fact, when there is a sufficient drop in the ability of water to solvate SiO_2 along the capillary length, the treatment with supercritical water can even lead to reduction of the capillary diameter through

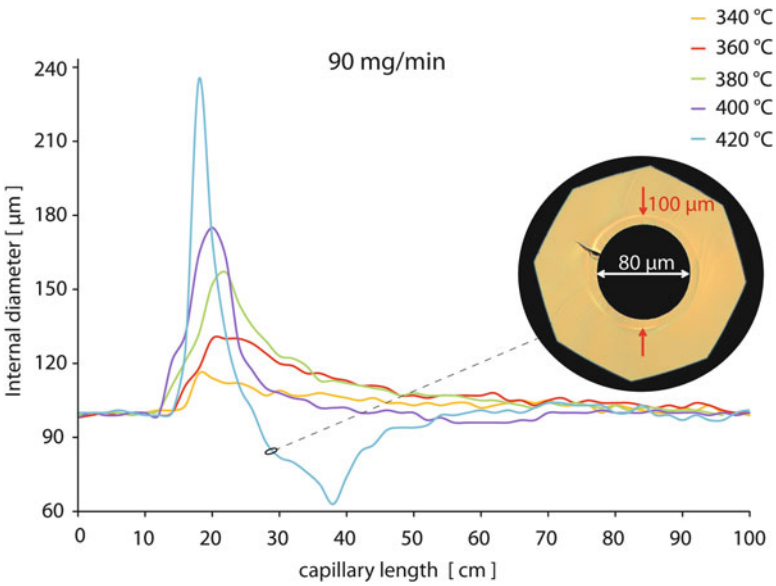

Fig. 8.9 Internal diameter profiles in fused-silica capillaries (nominal internal diameter = 100 µm) resulting from action of near-critical water at a 90 mg/min flow rate. Reprinted with permission from Karásek et al. (2013c). Copyright 2013 American Chemical Society

downstream re-deposition of the dissolved silica on the capillary wall from the upstream-generated aqueous solution. The re-deposited layer is firmly bound with the inner wall of the capillary and it does not show any apparent internal structure, at least when observed by the electron microscope.

As the flow of supercritical water through the capillary tends to produce changes in the internal diameter, a question naturally arises of how to limit the action of water just to roughening of the inner surface without producing any significant changes in the internal diameter of the capillary. Such a mode of operation is needed, e.g., when pre-treating the fused silica tubing to prepare a capillary chromatographic column or when the tubing is to be used for capillary zone electrophoresis. As it was mentioned above, the static mode of etching the capillary with a single charge of water has not lead to noticeable effects on the inner surface. Therefore, a viable way to the surface roughening obviously requires repeated periods of static leaching with fresh portions of supercritical water. To avoid the changes of internal diameter, the content of the capillary has to be replaced very rapidly to minimize any prolonged flow. This can be accomplished with a computer-controlled valve that can be opened for short periods of time down to 0.1 s. The multiple periods of static leaching with fresh portions of supercritical water, termed semi-dynamic mode of operation (Karásek et al. 2013c) thus make it possible to modify the inner surface roughness while preserving a uniform internal diameter of the capillary. Both the dynamic and the semi-dynamic mode of treating fused silica capillaries with supercritical water promise worthwhile benefits in the subsequent applications of the treated capillaries in analytical separation methods.

8.4 Water-Treated Fused Silica Capillaries and Their Applications in Food-Related Analysis

8.4.1 Capillary Isoelectric Focusing of Important Microorganisms in Tapered Capillaries

Together with isotachophoresis, isoelectric focusing belongs to the (analytical) separation methods that can concentrate the individual components above their respective concentrations in the original sample mixture. The method is only suitable for ampholytic molecules carrying both positive and negative charges depending on the functional groups contained in the molecule, and it capitalizes on the existence of the ampholyte's isoelectric point (p*I*) corresponding to the pH value at which the positive and negative charges are balanced and the ampholytic species carries no net electrical charge. An important portion of bioanalytes (e.g., amino acids, peptides and proteins) are of amphoteric nature. There are several variants of isoelectric focusing that differ in the format and/or medium of the separation space–gel isoelectric focusing, preparative (free flow) isoelectric focusing, and capillary isoelectric focusing (CIEF) (Righetti 2006; Silvertand et al. 2008; Koshel and Wirth 2012; Righetti et al. 2013). The latter variant is the subject here. At the start of the process, the capillary contains a suitably composed mixture of ampholytes including the components of the injected sample. Application of the voltage gradient along the capillary (~20 kV) then leads to gradual formation of a pH gradient and a simultaneous electromigration and focusing of the analytes to the positions where the local pH matches their respective p*I* values. The individual focused zones result from the outcome of the competing actions of electromigration forces and diffusion. In principle, the positions and concentration profiles of the individual zones could be obtained by scanning along the whole length of the capillary. However, a common experimental arrangement employs an optical (UV/Vis or fluorescence) detector at a fixed position making it necessary to mobilize the whole content of the capillary and to "push" it through the detection point, hopefully without disturbing the variance and resolution of the zones of individual analytes. There are several mechanisms available to mobilize the capillary content including a pressure gradient-driven mobilization, chemical mobilization, and mobilization employing the electro-osmotic flow. In applications to be described below, the latter mechanism has been employed. It should be noted that, unlike the parabolic velocity profile typical of the pressure gradient-driven flow, the flow profile in electro-osmotic flow is nearly rectangular (piston-like) with the ensuing minimum disturbance of the resolution of individual analyte zones. The formation of the pH gradient, migration and focusing of the individual analyte zones, and electro-osmotic flow-driven movement of the content of the capillary all occur simultaneously making it difficult to visualize the whole process by a simple graphic presentation.

In practice, the CIEF method makes it possible to separate the analyte pairs differing in their isoelectric points by as little as 0.01 pH unit. Considering

that the applicable range of pH spans across ~10 pH units, this results in the attainable separation capacity of about 1,000 individual species in a single run. In order to appreciate the full range of prospective applications it is important to note that, because of amphoteric nature of cell membranes, even single-cell organisms such as bacteria display their effective surface charges and can therefore be separated by electromigration techniques including CIEF (Armstrong et al. 1999; Shen et al. 2000; Rodriguez and Armstrong 2004; Kremser et al. 2007; Kostal and Arriaga 2008; Petr and Maier 2012; Šalplachta et al. 2012). The applications of CIEF to this purpose in this laboratory have involved the separations of a large number of microbial species and strains related to food processing and protection (Horká et al. 2009a) as well as to human health (Horká et al. 2003, 2006a, b, c, 2009b, 2011; Ruzicka et al. 2007), e.g., separations of plant pathogens of the *Clavibacter*, *Xanthomonas* and *Pseudomonas* genera (Horká et al. 2009a), separation of phenotypically indistinguishable *Candida* species (Horká et al. 2011), differentiation between biofilm-positive and biofilm-negative *Staphylococcus epidermidis* strains (Ruzicka et al. 2007) and separations of multiple microorganisms including *Candida* sp. (Horká et al. 2003, 2006a, b, c, 2009b), *Enterococcus faecalis* (Horká et al. 2003, 2006a, b, c), *Escherichia coli* (Horká et al. 2003, 2006a, b, c, 2009b), *Klebsiela pneumoniae* (Horká et al. 2006b), *Proteus vulgaris* (Horká et al. 2006b), *Saccharomyces cerevisiae* (Horká et al. 2006c), *Staphylococcus aureus* (Horká et al. 2006a), *Staphylococcus epidermidis* (Horká et al. 2003, 2006a, b, c, 2009b), *Streptococcus agalactiae* (Horká et al. 2006a, b) and *Stenotrophomonas maltophilia* (Horká et al. 2006a, c).

In fact, after the CIEF run, the individual separated and focused microbial species can be recovered from the capillary, cultivated on a cultivation medium, and subsequently characterized by employing mass spectrometry (Petr et al. 2009) with the mass spectra serving to identify the microorganisms studied. The reliability of identification can be improved by using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Demirev and Fenselau 2008; Carbonnelle et al. 2011). This option adds a powerful off-line identification dimension to the CIEF separation and focusing as the individual microbial species usually display their respective individual characteristic mass spectrometric fingerprints (Horká et al. 2010; Šalplachta et al. 2013).

Of particular importance in focusing electromigration methods are the internal diameter of the separation capillary and the profile of the internal diameter along the length of the separation capillary. In isotachophoresis, the effect of internal diameter of the separation compartment (fused silica capillaries had yet to arrive) had been recognized long ago (Verheggen et al. 1977) with the ensuing recommendation (Everaerts et al. 1979) and application (Dolník et al. 1985; Foret et al. 1990; Stegehuis et al. 1991) of a combination of large-bore and narrow-bore separation channels.

Later on, Šlais (Šlais 1994, 1995a, b) suggested that the separation efficiency in focusing electromigration methods would benefit from using a continuously tapered capillary instead of a combination of two conventional cylindrical capillaries of different diameters. Theoretical analysis of the problem of focusing in a moving pH gradient yielded the suitable profile of the internal diameter along the capillary length (Šlais 1994, 1995a, b, 1996). At the time, it had been somewhat difficult to validate the theoretical considerations because of the lack of a dependable method for reproducible preparation of tapered capillaries. The production method has now been available (Karásek et al. 2013c) and so it is possible to cut out from a SCW-treated capillary a section with the longitudinal profile of the internal diameter to match the profile suggested by theory (Šlais 1994, 1995b) and test the tapered capillary in a CIEF experiment (Šlais et al. 2013). Figure 8.10 indicates that, in a model mixture of p*I* markers (purpose-designed ampholytic dyes (Šlais and Friedl 1994; Šlais and Friedl 1995; Horká et al. 2001; Šlais et al. 2002)) and proteins, the use of tapered capillary really results in a significant improvement of the separation efficiency over that achieved with a conventional cylindrical capillary.

In a follow-up study to the successful test of tapered capillaries in CIEF of model mixtures, the tapered capillaries have been applied to the CIEF separation of several species of probiotic *Lactobacillus* bacteria (Horká et al. 2013a) that play important role in milk processing. This study enabled an unambiguous identification of the individual species in real samples of cow's milk, with the use of tapered capillaries leading to a significant enhancement of the separation capacity and separation efficiency of the CIEF analyses. Further, cow's milk was spiked with the cells of selected bacteria species, analyzed by CIEF in tapered capillary, the focused and detected cells were collected from the capillary, deposited on the cultivation medium, cultivated, and subsequently identified using MALDI-TOF mass spectrometry. A companion study (Horká et al. 2013b) was aimed to separate several bacteria species of the *Dickeya* genus. Some species of *Dickeya* are broad-host-range phytopathogens that can cause serious damage on important crops. Therefore, an efficient separation and reliable identification of the individual species of *Dickeya* is very important for phytosanitary protection. As illustrated by Fig. 8.11, the use of a tapered capillary again leads to an improved separation of several *Dickeya* bacteria species as compared to the separation accomplished in a conventional cylindrical capillary with a constant cross-section. In a similar way as that described in the separation of *Lactobacillus* bacteria, the individual separated species of *Dickeya* bacteria can subsequently be identified employing the MALDI-TOF mass spectrometry.

The two example separations of bacteria by CIEF in tapered capillaries (Horká et al. 2013a, b) illustrate the indirect way in which supercritical water can be useful to improve the efficiency of electromigration analytical separations related to food analysis and food control.

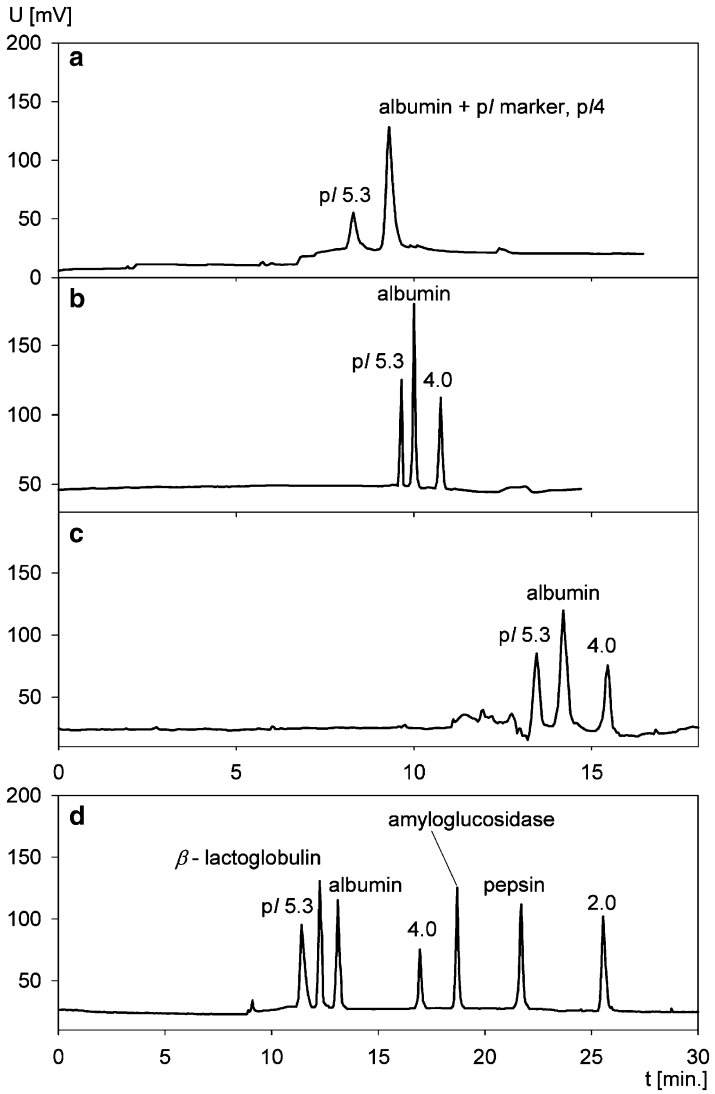

Fig. 8.10 Influence of the capillary geometry, cylindrical (**a**, **c**) and tapered (**b**, **d**), on CIEF separation of p*I* markers and proteins in the pH gradient 2.0–5.3. Conditions: fused-silica capillaries: cylindrical – 100 µm ID, 360 µm OD, 87.6 cm length (67.6 cm length to the detection window + 20 cm toward the electrode vial); tapered – capillary ID from 170 µm at the inlet of the capillary to 100 µm at the detection window, taper length 45 cm + 20 cm of 100 µm ID toward the electrode vial. Background electrolyte: 2×10^{-2} mol L^{-1} phosphate buffer from pH 2 to pH 10; siphoning injection ($\Delta h = 10$ cm, 5 s); applied voltage (−)20 kV; neutral marker of electroosmotic flow, thiourea; UV detection, 235 nm. The catholyte, 40×10^{-2} mol L^{-1} NaOH, the anolyte, 0.1 mol L^{-1} H_3PO_4; sample composition—segment of spacers dissolved in the catholyte, t_{inj}, 25 s, segment of carrier ampholytes, 5 %(w/v) of synthetic carrier ampholytes, Biolyte, pH 3–10, ampholyte pH 3–4.5 and pH 2–4 (1:2:5), t_{inj}, 35 s; UV detection, 280 nm; (**a**) sample: p*I* markers p*I* 5.3 and 4.0 (25 µg mL^{-1} of each), albumin, 50 µg mL^{-1}, dissolved in water; t_{inj}, 10 s (Δh, 20 cm); (**b**) see A, t_{inj}, spacers, 10 s, sample 10 s and carriers 17 s; (**c**) see A, 0.5 %(w/v) PEG 10 000 was dissolved in the catholyte and the anolyte; (**d**) see A, 0.3 %(w/v) PEG 10 000 and 5 %

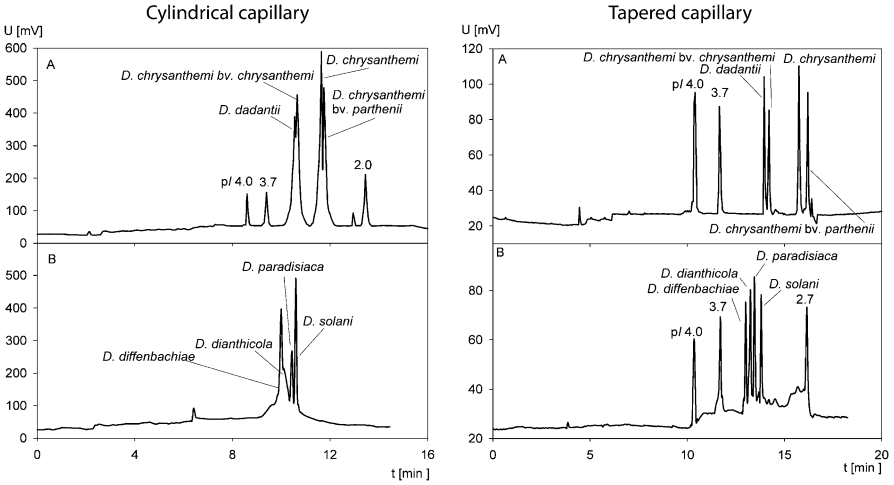

Fig. 8.11 Capillary isoelectric focusing separations of two samples (**a** and **b**) containing several species of *Dickeya* bacteria with mutually close p*I* values and isoelectric point markers in 2.0–4.0 pH gradient in the cylindrical and the tapered fused-silica capillary. Cylindrical capillary: The examined bacteria were resuspended in demineralized water with addition of 1 % (w/v) PEG 10,000 to concentration of 1×10^8 cells mL^{-1}. Tapered capillary: The examined bacteria were resuspended in demineralized water with addition of 1 % (w/v) PEG 10,000 to concentration of 1×10^7 cells mL^{-1}. Adapted with permission from Horká et al. (2013b). Copyright 2013 American Chemical Society

8.4.2 *Water-Treated Cylindrical Capillaries for Preparation of Monolithic Silica Columns*

During the last two decades, monolithic chromatographic columns have become established as an ambitious complement of particle-packed columns, primarily in the chromatographic methods employing liquid mobile phases. In the monolithic columns, the particle-packed bed has been replaced with a single-piece structure (porous rod) featuring suitable distribution of the average size of flow-through pores (Svec and Fréchet 1992; Guiochon 2007; Unger et al. 2008). In general, the most important benefit of a monolithic column usually comes from relatively higher permeability and the resultant lower pressure drop as compared to those typical of a particle-packed column. It is important to keep the column pressure drop low because a large pressure drop is associated with the effects of expansion of the

Fig. 8.10 (continued) (v/v) EtOH were dissolved in the catholyte and the anolyte; sample: p*I* markers p*I* 5.3, 4.0, 2.0 and proteins, β-lactoglobulin, albumin, amyloglucosidase and pepsin, 50 μg mL^{-1} of each, t_{inj}, 10 s; rinsing procedure, EtOH for 5 min, and then back-flushed with the catholyte for 1 min; *t*, migration time [s]. Reprinted with permission from Šlais et al. (2013). Copyright 2013 American Chemical Society

mobile phase and dissipation of viscous heat. These adverse effects detract from the column efficiency and tend to gain in magnitude as the scale of the column increases from analytical to preparative. In capillary column format, an additional benefit of monolithic columns derives from the absence of the column-end frits that are needed to retain the particle bed in packed columns and may cause problems compromising the column efficiency (e.g., spurious adsorption of the analytes).

As regards the build-up material of the monolith skeleton, the monolithic columns can be divided in two broad classes: (a) rigid organic polymer-based monolithic columns (Svec and Fréchet 1992; Urban and Jandera 2008; Svec 2010; Arrua et al. 2012) and (b) silica-based monolithic columns (Nakanishi and Soga 1992; Minakuchi et al. 1996; Tanaka et al. 2002; Siouffi 2003; Núñez et al. 2008). Each of the two column classes has its own strengths and weaknesses. The polymer-based monolithic columns are relatively easy to prepare and relatively robust with respect to the allowable pH of the liquid mobile phase. In turn, the silica-based monolithic columns are more difficult to prepare, their usable pH range is usually limited to pH < ~8 but they are capable of producing higher efficiencies than those typical of organic polymer-based monolithic columns. The following text in this section will only be concerned with silica-based monolithic columns in the capillary format.

Silica-based monoliths designated for capillary liquid chromatography are prepared *in situ* (inside the fused silica capillary) by using a sol–gel process involving sequential acid hydrolysis and polycondensation of alkoxy silicon derivatives (most often tetramethoxysilane) in the presence of porogen (usually polyethylene glycol) at elevated temperature (Nakanishi and Soga 1992; Minakuchi et al. 1996; Motokawa et al. 2002; Hara et al. 2006; Planeta et al. 2010). The process also involves the use of urea (Puy et al. 2007); during the thermal treatment following the sol–gel process, urea decomposes and releases ammonia that plays a role in tailoring the pore-size distribution of the resultant silica monolith. After a careful rinsing and calcination at ~320 °C, the bare silica monolithic column can either be used directly for chromatographic separations or it may go through a chemical modification to form a retentive layer on the monolith surface and provide the column with the desired selectivity. The scanning electron micrograph of a transversal cut through the silica monolithic column (Moravcová et al. 2012) shows a typical porous structure with a defect-free bonding of the monolith to the inner wall of the capillary (Fig. 8.12).

It has been shown that nonuniform-diameter capillaries with an internal taper can be employed to enhance the separation efficiency of amphoteric analytes in CIEF (see Sect. 8.4.1). However, as discussed in Sect. 8.3, the treatment of fused silica capillaries with supercritical water in semi-dynamic mode can produce the inner surfaces of diverse morphologies and varying degrees of roughness while keeping the internal diameter of the capillary essentially constant. This type of treating the fused silica capillaries with SCW can potentially be useful in preparation of monolithic silica-based capillary columns.

For example, Fig. 8.13 shows a scanning electron micrograph of the inner surface obtained by semi-dynamic mode of treating the capillary with SCW

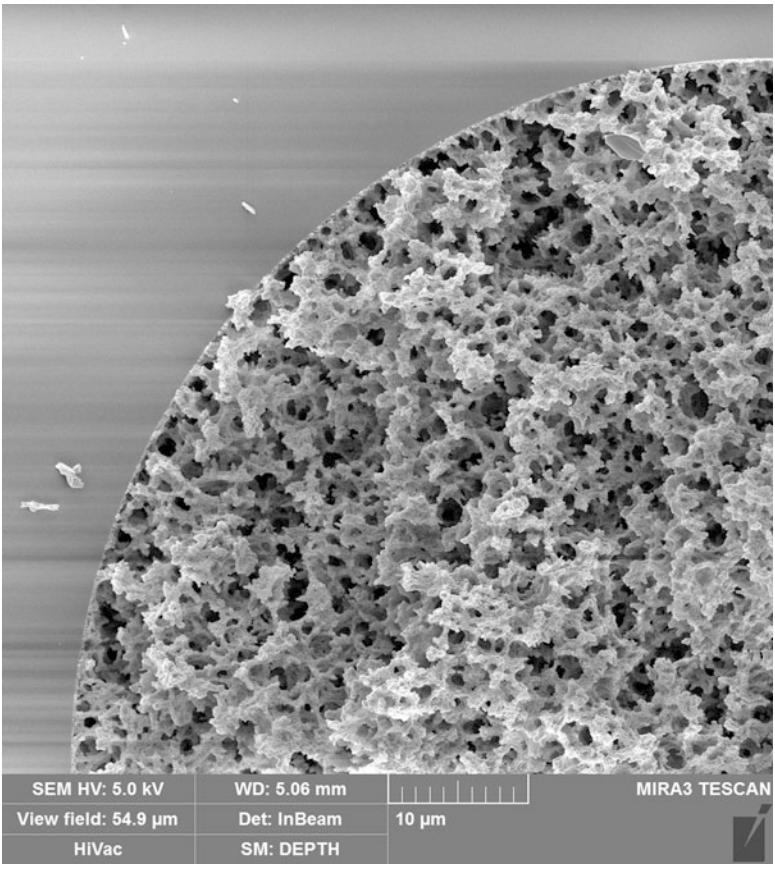

Fig. 8.12 SEM photograph of a cross-cut through a monolithic-silica capillary column (100 µm i.d.) showing a typical structure of the silica monolith. Reprinted with permission from Moravcová et al. (2012). Copyright 2012 Elsevier Science

(Karásek et al. 2013c). Compared to the original smooth inner surface of the capillary, the crest-and-gorge-like surface with the "crests" oriented in the perpendicular direction to the capillary axis can be useful in silica monolith preparation inside the capillary as the penetration of the monolith into the "gorges" on the inner surface helps to secure the monolith against the axial movement in the capillary. This feature is rather helpful because, if the inner surface retained its original smoothness, the pressure of the liquid mobile phase could easily shoot the monolithic core out of the column as a thin fiber. Besides, in addition to roughening the inner surface to provide a mechanical hindrance to the axial movement of the monolith, the treatment with SCW also obviously increases the inner surface area of the capillary wall. The latter enhances the number of monolith-to-wall Si–O–Si links which further stabilize the monolithic core inside the capillary.

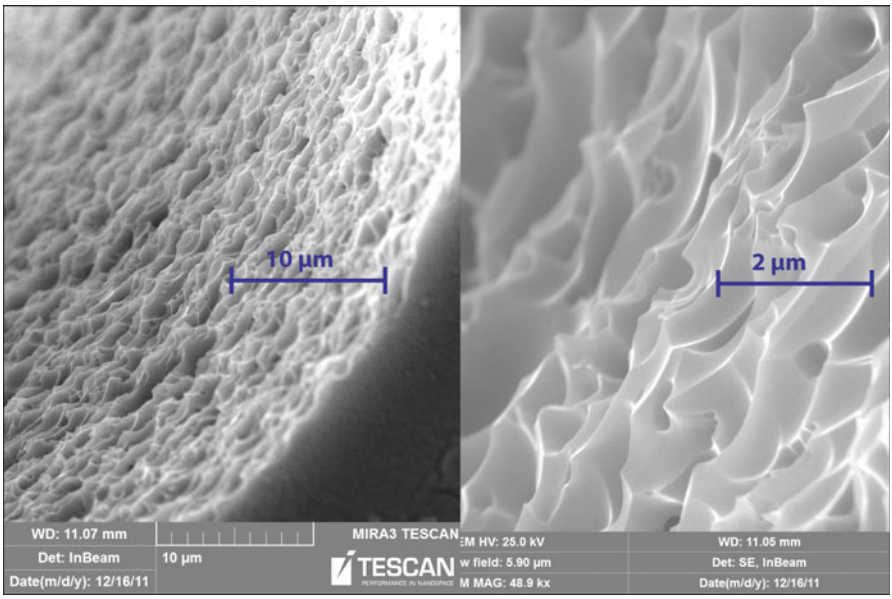

Fig. 8.13 SEM photograph of the inner surface of supercritical water-treated fused-silica capillary. Operating conditions: 400 °C, 32 MPa, semidynamic mode, 20 replacements of water in the capillary, 60 s residence time of a single batch of water. Reprinted with permission from Karásek et al. (2013c). Copyright 2013 American Chemical Society

What follows is a short illustration of the use and application power of monolithic silica columns. In liquid chromatography, the monolithic silica-based capillary columns can in principle be used with "bare" surface of the silica monolith in the normal-phase chromatographic mode. Usually, however, the surface of the silica monolith has been modified by grafting to it some suitable organic moieties in order to tailor the desired selectivity and/or the retention mechanism of the column. The modifications may certainly involve grafting of C8 or C18 alkyl chains to result in the monolithic counterparts of C8 or C18 discrete particle packings commonly used in reversed-phase liquid chromatography. As an ever increasing portion of liquid chromatographic analyses serve the needs of molecular biology, biochemistry and related disciplines including food science and clinical chemistry, there is a pressing need for columns with specific selectivity features to separate polar compounds of interest in these fields. An example of a chromatographic method to address these applications is hydrophilic interaction chromatography (HILIC) (Alpert 1990).

HILIC has recently become frequented in the separations of polar compounds (e.g., pharmaceuticals, nucleosides, or nucleotides) in aqueous-organic mobile phases rich in organic solvents (usually acetonitrile) (Jandera 2011; Buszewski and Noga 2012; Gama et al. 2012). The mechanism of analyte retention in HILIC involves the acidobasic proton donor–acceptor and dipole–dipole interactions of the analyte with both the surface of the stationary phase and with the liquid (mostly water) occluded on the surface in the diffusion layer. Monolithic silica can be turned

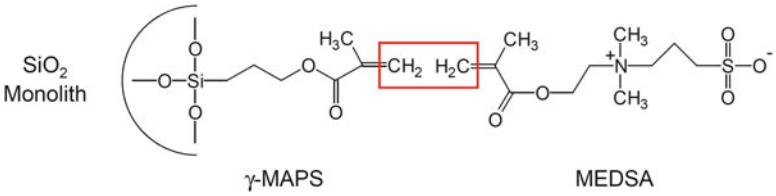

Fig. 8.14 Scheme of modification of silica monolith with γ-MAPS and MEDSA to a zwitterionic sulfoalkylbetaine stationary phase suitable for HILIC

into a HILIC stationary phase through modification of the monolith surface with a suitable organic moiety (Ikegami et al. 2006, 2008; Horie et al. 2007; Malerod et al. 2013), e.g., with a zwitterionic sulfobetaine (Wohlgemuth et al. 2010). An example of the monolith modification (Moravcová et al. 2012) involves a two-step process. In the first step, the silica monolith is reacted with 3-trimethoxysilylpropylmethacrylate (γ-MAPS); in this step, the trimethoxysilyl terminus of γ-MAPS becomes bonded to the monolith surface whereas the methacrylate terminus of γ-MAPS sticks out. In the second step, the vinyl group in the methacrylate terminus of γ-MAPS undergoes a thermally initiated radical co-polymerization with the vinyl group of [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide (MEDSA) as shown schematically in Fig. 8.14. As a result, the original bare surface of the silica-based monolith is turned into a surface bearing grafted chains terminated with zwitterionic sulfobetaine moieties. Thereby, the silica-based monolith is turned into a chromatographic stationary phase suitable for HILIC. Figure 8.15 (Moravcová et al. 2012) illustrates the isocratic separations of a mixture of UV detectable analytes including nucleic acid bases (adenine, cytosine, thymine, and uracil), nucleosides (adenosine, cytidine, guanosine, 5-methyluridine, and uridine), and deoxynucleosides (2-deoxyadenosine, 2-deoxyuridine, 2-deoxycytidine), with toluene serving to mark the column hold-up time. Comparison of the two chromatograms, namely A and B in Fig. 8.15, confirms that the separation efficiency of the HILIC column provides a very significant improvement over the separation efficiency of the bare (unmodified) silica-based monolithic column. On the HILIC columns, all the analytes shown in Fig. 8.15 are baseline-separated, and the reproducibility of separation is also much better than that on the bare silica column.

It should be noted, however, that the example separations in the zwitterionic sulfobetaine-modified column have been obtained with the source fused silica capillary pre-treated with conventional agents rather than with supercritical water. Nevertheless, the example separations have been shown here to illustrate the separation power of modified monolithic silica-based capillary columns as regards polar compounds of biological relevance. The column used for the analysis shown in Fig. 8.15 (chromatogram B) had been operating for about half a year. At the end of its usable lifetime, a small part of the monolithic core broke away and the column's efficiency deteriorated considerably. The prospective use of supercritical water as a treatment agent for the source fused silica capillary is expected to improve the robustness and

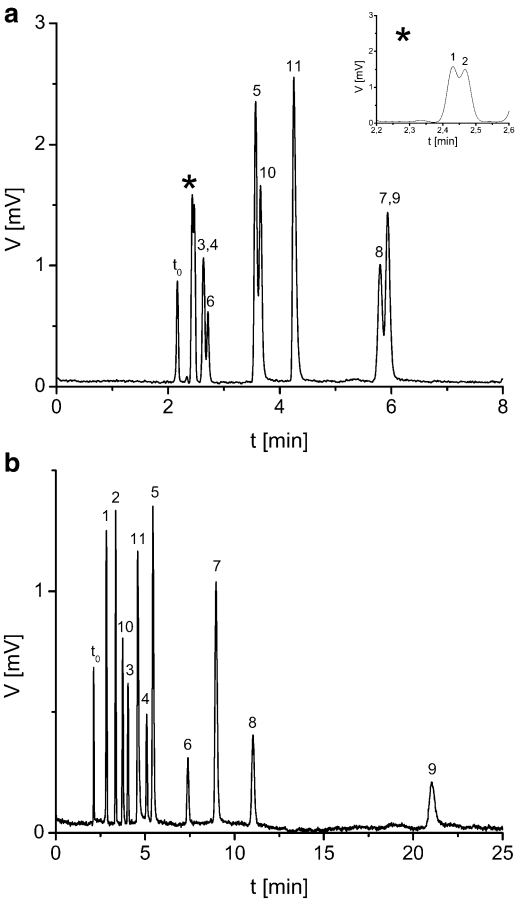

Fig. 8.15 Comparison of isocratic elution on bare silica monolithic (**a**) and sulfoalkylbetaine monolithic (**b**) capillary columns. Mobile phase: 95 % (v/v) acetonitrile/ 50 mM ammonium formate, pH = 4.5, flow rate 0.5 μL/ min; detection: UV 210 nm; sample: toluene (column hold-up time marker), thymine (1), uracil (2), 2-deoxyuridine (3), 5-methyluridine (4), adenosine (5), uridine (6), cytosine (7), 2-deoxycytidine (8), cytidine (9), 2-deoxyadenosine (10), adenine (11), and adenosine (12). Reprinted with permission from Moravcová et al. (2012). Copyright 2012 Elsevier Science

stability of the monolithic column rather than to produce a significant enhancement of the separation efficiency.

Conclusion

Because of full compatibility of water at ambient conditions with food processing, a general, direct use of PHW for extraction and/or chromatography of substances relevant to food science would certainly be highly desirable. However, the direct applicability of PHW to these purposes is rather limited by the stability of the target compounds in aqueous environment under the operating temperature in PHWE. Nevertheless, this certainly does not imply that very hot water is useless in applications related to food science and food processing because, in these fields, even a highly aggressive

(continued)

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medium such as near- and supercritical water can indirectly be useful through the analytical separation devices involved.

It has been illustrated above that a judicious application of high-temperature water to etch fused silica surfaces can lead to enhanced performance of fused silica-based devices for analytical separation methods. Moreover, owing to the use of pure water as the treatment agent, the procedures of treating the inner surfaces of fused silica capillaries are invariably greener as compared to those employing conventional agents. To the date of this writing, the first verified benefits of sub- and supercritical water in this respect concern the preparation of tapered capillaries and their use to enhance the separation efficiency in the capillary isoelectric focusing of several bacterium species relevant to food processing. Further investigations aimed on the use of SCW-treated capillaries of constant internal diameter in preparation of chromatographic columns and on the use of hot water to prepare microchannel structures in glass supports for lab-on-a-chip applications have been under way. The resultant gains derived from the ensuing improvements in separation efficiency may be useful in all applications of analytical separation methods, with those related to food science, food processing and food diagnostics making no exception.

Acknowledgments Financial support of the Czech Science Foundation (Projects P206/11/0138, P503/11/P523 and P106/12/0522), of the Ministry of Interior of the Czech Republic (Projects VG20102015023 and VG20112015021), and of the Academy of Sciences of the Czech Republic (Institutional Support RVO:68081715) is gratefully acknowledged.

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Part III
Current and Future Applications

Chapter 9
Supercritical Fluid Processing for the Recovery of Bioactive Compounds from Food Industry By-Products

M. Esra Yener

9.1 Introduction

Today consumers are highly aware of the close relationship between nutrition and health and they want to include health-promoting ingredients in their diets. Foods fortified with nutraceuticals, or functional foods in other terms, are expected to be the new food category with an expanding demand by future generations. Therefore, food scientists focus their effort on the development of new products with improved nutritional profiles. Natural ingredients recovered from agro industrial by-products have specific dietary and functional properties and can be utilized effectively to develop this new food category (Oreopoulou and Tzia 2007).

Food industries are mainly based on natural plant-derived agro-products and animal products. The major food industries include predominantly fruit and vegetables, spices, sugarcane, bakeries, confectioneries, oilseeds, beverages, milk and milk based products, egg, meat and seafood. Food processing in these industries results in a range of wastes and by-products according to the types of raw material processed for deriving food and the respective processing technologies employed (Muragan et al. 2013).

Use of green processing is essential for recovering bioactive compounds from food industry by- products. Supercritical fluid extraction (SFE) is a superior tool for this purpose. It enables effective and selective extraction of bioactive compounds from natural matrices by changing extraction conditions without or with limited use of cosolvents as ethanol. Removal of organic solvents from the extracts is also eliminated by the use of supercritical fluids. Supercritical carbon dioxide ($SCCO_2$) is a perfect fluid for the extraction of light, heat and air sensitive biomaterials because of its low critical pressure (7.38 MPa) and temperature (31.2 °C). Use of

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cosolvents is required to increase the solubility of polar bioactive compounds in $SCCO_2$.

One third of the supercritical fluid processing applications are in the area of food and agriculture (Herrero et al. 2006). Major applications include the extraction and fractionation of essential oils, oils and bioactive compounds like antioxidants from natural matrices (Machado et al. 2013; Herrero et al. 2006; Temelli 2009; Catchpole et al. 2009b; Reverchon and de Marco 2006). Value added products rich in bioactives are obtained by supercritical fluid processing (Reverchon and de Marco 2006; Temelli 2009; Catchpole et al. 2009b).

Recently, recovery of bioactive compounds from food industry by-products has been gaining importance (Herrero et al. 2013; Wijngaard et al. 2012; Herrero et al. 2010; Pereira et al. 2010). This chapter outlines $SCCO_2$ extraction of specialty oils rich in bioactive compounds, fractionation of specialty oils or by-products to enrich the bioactive compounds, SFE of carotenoids and, either SFE or subcritical fluid extraction (SCFE) and, supercritical antisolvent extraction (SAE) and fractionation (SAF) of polyphenols.

9.2 Bioactive Compounds in Food Industry By-Products

Processing of fruits and vegetables results in high amounts of waste materials such as pomace, peels, seeds, stones or kernels (Oreopoulou and Tzia 2007). Beverage industry the majority of which consists of wine and fruit juices utilizes grape, apple, peach, apricot, cherries, berries, pomegranate, citrus fruits, mango, pineapple, banana, guava, papaya in large amounts (Kumar and Chandrasekaran 2013). Pomace and peels of these fruits are rich in antioxidants, pigments -carotenoids, polyphenols- (Kao and Chen 2013) while seeds, stones or kernels are rich in mono- (MUFAs) or polyunsaturated fatty acids (PUFAs).

Oil industry is a major part of vegetable, oilseed and legume (soybean) processing. The traditional press extraction method, as well as the continuous three-phase decanter process, which are most widely used for the production of olive oil, generates two by-products. A solid by-product called either crude olive cake or olive husk, and an aqueous by-product called olive mill wastewater (Tsagaraki et al. 2007; Krishna and Chandrasekaran 2013). Oil cakes or oil meals are by-products obtained after oil extraction from the seeds (Chandrasekaran and Shine 2013). Deodorizer distillate (DOD) is a by-product of deodorization which is the last major step of vegetable oil refining process. Deodorizer distillates are excellent sources of sterols, tocopherols and squalene.

Wheat, rice, corn, millet, oats, rye, and barley are the most important grains for human consumption (Russ and Schnappinger 2007). Wheat milling results in large quantities of bran and germ as by-products (Krishna and Chandrasekaran 2013). Wheat germ oil is rich in PUFAs and is highly valuable with a low ω-6 to ω-3 fatty acids ratio of 7. Wheat germ contains important bioactive compounds such as antioxidants and sterols. Antioxidants of wheat germ include tocopherols,

tocotrienols (together abbreviated as tocols and summarized under the term vitamin E), phenolics and carotenoids. Wheat germ (consequently wheat germ oil) is the richest source of tocopherols and plant sterols (Schwartz et al. 2008). Ferulic acid and vanillic acid in free form and glycoflavones are the phenolics reported in wheat germ. The most abundant carotenoids in wheat germ oil are found to be lutein, zeaxanthin and β-carotene (Panfili et al. 2003). Wheat extracts are reported to show strong antioxidant activities higher than those of vitamin E, vitamin C, and the well-known synthetic antioxidants (Yu et al. 2002).

Rice bran is another valuable by-product of milling industry (Krishna and Chandrasekaran 2013). When rice is milled to produce white rice, the outer layers of the rice kernel are removed. These layers include the hull, the germ, and the bran. Depending on the rice type and applied stabilization technique, rice bran contains on average (10–23) % oil (Sparks et al. 2006). Crude rice bran consists of (0.5–1) wt % oryzanols that are derivatives of phytosterols and ferulic acid. The strong antioxidant property of γ-oryzanols has been widely recognized (Wang et al. 2008).

Seafood generally refers not only fish but marine habitats and shellfish. The species of fish that are usually processed by seafood processing companies include cod, hake, haddock, tuna, herring, mackerel, salmon and pollock. Shellfish are members of four different groups: The first group includes crustaceans such as crab, shrimp/prawn, lobster, crayfish, krill, langoustines. The second group includes *behalves* such as clams, oysters, whelks, scallop, mussels and cockles, *unihalves* such as snails, abolone, conch, and *cephalopods* such as squids, cuttlefish and actopods. The third and fourth groups include urchins and sea cucumbers, and jellyfish, respectively (Suresh and Prabhu 2013).

By-products generated during seafood processing typically range between (20 and 60)% of the starting raw material. For fish such as tuna, cod, mackerel, anchovy and herring, major amounts of residues are represented by offals, head and tail (27 % of the fish) collected through eviscerating and filleting process. Skin, bones, blood and frames are the second major residue (25 % of the fish) collected along skinning and cutting process. Conventionally, these by-products are processed into low-value products such as fish meals, fish oil and natural fertilizers or discarded. Fish meal is by far the most valuable non-edible commodity produced from seafood processing by-products. Fish oil can have non-edible and edible applications; the latter are essentially used for the production of margarine and shortening (Ferraro et al. 2010).

The most important sources of ω-3 PUFAs are indeed fish oils of species such as sardine, mackerel, cod, shark and menhaden with PUFA levels of 30 % which makes them commercially interesting raw materials to prepare PUFA concentrates. However, seafood processing by-products are also good sources of carotenoids besides many other novel and valuable components. Oil can be extracted from seafood processing by-products such as head, gut and liver of various species including shark, salmon, cod, catfish, herring and carps (Suresh and Prabhu 2013). The highest concentration of bioactive compounds is generally in the parts of the marine organisms that are discarded. The present concentrations can attain upto 80 % of the by-product in question especially in the case of lipids in cod liver.

Shark liver oil is also rich in PUFAs and it is the main source of squalene. Salmon head, which represents the main salmon processing by-product is also regarded as a good source of PUFAs (Ferraro et al. 2010).

Shellfish processing by-products are head, shells and tails. The concentration of desirable ω-3 PUFAs are reported to be high in shrimp heads (171 g/g dried material) and shrimp by-products (137 mg/g dried material) compared to that of shrimp muscle (16 mg/g dried material) (Amiguet et al. 2012). Crustaceans processing by-products are rich in carotenoids. Astaxanthin is a carotenoid which represents (74 to 98) % of the total pigments in crustacean shells. Being a precursor and an antioxidant make astaxanthin a widely sought for food and medical applications. It possesses an antioxidant effect greater than β-carotene as well as vitamin C and E (Ferraro et al. 2010).

Of the large variety of bioactive compounds present in natural sources, this chapter focuses only on PUFAs, tocopherols, phytosterols, squalene, carotenoids and polyphenols.

9.2.1 Polyunsaturated Fatty Acids

PUFAs contain two or more double bonds in their carbon chain. Most PUFAs are essential fatty acids and have to be provided to the body through the diet. They are usually classified as ω-3 and ω-6, depending on the position of the first double bond from the methyl end of the carbon chain. α-linolenic acid (C18:3, ALA), eicosapentaenoic acid (C20:5, EPA), docosapentaenoic acid (C22:5, DPA), and decosahexaenoic acid (C22:6, DHA) are examples of ω-3 PUFAs, whereas linoleic acid (C18:2, LA) and γ-linolenic acid (C18:2, GLA) are examples of ω-6 PUFAs (Temelli et al. 2008). The parent ω-3 fatty acid is ALA and the parent ω-6 fatty acid is LA. ω-6 fatty acids like arachidonic acid (C20:4, AA) can be synthesized by humans from LA, and ω-3 fatty acids, as EPA, DPA, DHA from ALA; however, the conversion of ALA in EPA, DPA and DHA is low and these ω-3 fatty acids are considered as essential fatty acids too (Rubio-Rodríguez et al. 2010).

The main source of EPA, DPA, and DHA are fish oils. Specialty oils of plant origin are rich in LA, ALA and GLA. An ideal intake ratio of ω-6 to ω-3 fatty acids is between 1:1 and 4:1. However, most people obtain these fatty acids at a ratio of (10:1 to 25:1) since it is rarely below 10 in most of the food. In wheat germ, cod liver, salmon and sardine oils this ratio is 7, 0.04, 0.03 and 0.07, respectively (Rubio-Rodríguez et al. 2010; Gelmez et al. 2009).

ω-3 PUFAs, especially EPA and DHA, play several roles in human health relating to nutritional benefits, coronary heart diseases, hypertension, diabetes, rheumatoid arthritis, development of brain, vision and reproductive systems, cancers and mental depression (Létisse and Comeau 2008).

9.2.2 Tocopherols

Tocopherols and tocotrienols make up the tocols family of vitamin E compounds, which must be obtained from the diet because humans cannot synthesize them. Tocols are found in specialty oils as rice bran and wheat germ oils, in oil industry by-products as oil meals and oil DODs. The difference between tocopherols and tocotrienols lies in the phytyl chain attached to a chromanol ring: the phytyl chain is saturated in tocopherols, whereas the phytyl chain in tocotrienols has three double bonds. These compounds represent a group of four isomers with varying numbers and position of methyl groups on the chromanol ring: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. Although all of these tocol isomers are absorbed through the intestine in the human body, it is believed that only α-tocopherol contributes toward meeting the human vitamin E requirement (Temelli et al. 2008).

Tocopherols and tocotrienols are the major fat-soluble antioxidants. They can scavenge free radicals in the body, thereby preventing them from damaging cell membranes and genetic material and changing the character of fats and proteins.

9.2.3 Phytosterols

The main sterols in plant materials are sitosterol, campesterol, and stigmasterol (Temelli et al. 2008). They are mainly found in the specialty oils from by-products as cherry seed oil and rice bran oil, and oil DODs. Over the years, it has been well established that a high dietary intake of phytosterols lowers blood cholesterol levels by competing with dietary and biliary cholesterol during intestinal absorption.

Oryzanols are derivatives of phytosterols and ferulic acid. γ-oryzanols are a mixture of ferulic acid esters of sterol and are found in rice bran. Furthermore, γ-oryzanols have a strong antioxidant property and reduce the plasma cholesterol (Wang et al. 2008).

9.2.4 Squalene

Squalene is a lipid that was originally obtained from shark liver oil. It is also found in olive, palm, and wheat germ oils (Temelli et al. 2008) and DODs. A number of animal studies showed that dietary squalene has distinct anticarcinogenic effects. It was shown that squalene presents inhibitory action in carcinogenesis models of skin, colon and lung cancer.

9.2.5 Carotenoids

Carotenoids represent a group of over 600 fat-soluble pigments. These pigments are responsible for the bright yellow, orange, and red colors of fruits, roots, flowers, fish, invertebrates, birds, algae, bacteria, molds, and yeast. Some carotenoids are also present in green vegetables, where their color is masked by chlorophyll. Carotenoids are typically divided into two classes: carotenes, which are C40 polyunsaturated hydrocarbons, and xanthophylls, oxygenated derivatives of carotenes. Carotenoid compounds are colored due to their high level of conjugated double bonds, which also makes them quite unstable. Indeed, each conjugated double bond can undergo isomerization to produce various *trans*/*cis* isomers, particularly during food processing and storage. About 10 % of carotenoids are called "provitamin A", indicating that they possess at least one unsubstituted β-ionone ring that can be converted into vitamin A (Temelli et al. 2008). The two main carotenoids that have been extensively studied are β-carotene and lycopene. In terms of by-products, carotenoids are mainly present in fruit and vegetable pomaces as carrot, apricot and tomato pomaces. Of all carotenoids, β-carotene has the highest provitamin A activity, approximately twice that of α- and γ-carotene. Lycopene, although lacking provitamin A activity, is known to be one of the most potent antioxidants among the digestible carotenoids. Its highly conjugated molecular structure is responsible for the bright red color of ripe tomatoes as well as the pigmentation of watermelons, pink grapefruits, apricots, and papayas. Astaxanthin is a pigment that belongs to the family of xanthophylls, the oxygenated derivative of carotenoids whose synthesis in plants derives from lycopene. It is the major pigment in crustacean shells. In addition to its effect on color, one of the most important properties of astaxanthin is its antioxidant properties which have been reported to surpass those of β-carotene or even those of α-tocopherol (Higuera-Ciapara et al. 2006).

9.2.6 Polyphenols

Polyphenols in food are classified as phenolic acids, flavonoids, lignans and stilbenes. Flavonoids are divided into six sub-groups as flavanols, flavons, flavanones, isoflavones, flavanols and anthocyanins. Flovanoles consist of monomer cathechins and polymer proanthocyanidins also known as tannins (Halvorsen et al. 2002; Manach et al. 2004; Bueno et al. 2012).

Polyphenols act as antioxidants; they scavenge free radicals which are responsible for serious diseases and for the oxidation of lipids, proteins, and DNA. Studies have revealed that polyphenols have several biological activities as antibacterial, antiinflammatory, antiallergic, immunologic, antiulcer, antitumor, anticancerogenic, antithrombotic, proapothetic, and vasodilator activities. They have a preventive effect on many diseases related with oxidative stress as diabetes,

hypertension, osteoporosis, cancer, arthritis, cardiovascular and neurological diseases (Bueno et al. 2012). In addition to health benefits, the supplementation of food products with antioxidants delays the formation of off-flavors and rancidity and extends the shelf life of the product.

Recovery of polyphenols from natural products and food industry by-products has been gaining importance because natural antioxidants are preferred over synthetic antioxidants (Schieber et al. 2001). It was shown that polyphenols have higher antioxidant activities than the other natural antioxidants such as vitamin C, vitamin E and carotenoids. Fruits, vegetables, nuts, fruit pomaces and seeds are rich in polyphenols (Moure et al. 2001).

9.2.6.1 Antioxidant Evaluation Protocols

The methods for evaluation of antioxidative action should be based on the identification of different antioxidative mechanisms under variable conditions; reflecting the multifunctional properties of antioxidants in both physiological and food-related oxidative processes. The procedure for the application of antioxidants to food, including evaluation of health benefit, includes basically four steps. The first step is quantification and identification of phenolic compounds in the food product. The second step concerns quantification of the radical scavenging activity of different antioxidants using more than one method and considering the effect of solvent on the antioxidant mechanism. The third step is the evaluation of the ability of the antioxidant to inhibit or halt lipid oxidation in relevant model systems. The last step depends on the aim of the study. For food applications, storage experiments using actual antioxidants incorporated in the food product of relevance are mandatory. For human intervention, evaluation of dietary antioxidant effect in the human body is necessary (Becker et al. 2004).

Quantification of total phenolic content (TPC) by the Folin-Ciocalteu method is based on the number of phenolic groups present in the sample such as gallic acid. This method is based on measuring the color change caused by reduction of the Folin-Ciocalteu reagent by phenolates in the presence of sodium carbonate. The concentration of phenolics is reported as mg gallic acid equivalent (GAE)/g (Adil et al. 2007, 2008; Gelmez et al. 2009).

Quantification of the radical scavenging activity includes several different assays which are classified in three major groups. The first group includes classical assays for detection of electron transfer antioxidant activity which are TEAC (Trolox Equivalent Antioxidant Capacity) and FRAP (Ferric Reducing Power) assays. The TEAC assay relies on the reduction of the colored cation radical of 2, 2′-azanobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{+\cdot}$). The FRAP assay measures the antioxidant capacity by the reduction of tripyridyltriazine complex to the blue ferrous complex (Becker et al. 2004). The second group includes the assays for detection of scavenging of stable radicals. Scavenging of 1, 1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) is extensively used. The assay is based on the color change caused by reduction of the $DPPH^{\bullet}$ radical which is determined by

measuring absorbance at 515 nm. The antioxidant activity (AA) is reported either as mg scavenged $DPPH^{\bullet}$/g sample (Gelmez et al. 2009) or as antiradical efficiency (AE) which is defined as $1/EC_{50}$ (Adil et al. 2007, 2008). EC_{50} is the efficient concentration of the sample to decrease the initial $DPPH^{\bullet}$ concentration by 50 %. The third group includes assays for detection of scavenging of short-lived radicals which are ORAC (Oxygen Radical Absorbance Capacity) and FRBR (Fenton Reaction Based Radical) assays. The ORAC assay is based on the quenching of fluorescence from the protein β-phycoerythrin by radicals. In the FRBR assay detection of hydroxyl radicals after reaction with the span trap 5, 5-dimethyl-1-pyroline-N-oxide (DMPO) has been used for the measurement of antioxidative capacity (Becker et al. 2004).

9.3 Supercritical or Subcritical Fluid Extraction and Fractionation

9.3.1 Extraction of Specialty Oils

Specialty oils are low volume, high value added products which are rich in bioactive compounds. They are obtained from nuts (acorn, almond, hazelnut, peanut, pecan, pistachio, walnut), seeds (apricot, borage, cherry, echium, evening primrose, flax, grape, hiprose, pumpkin, rosehip, sea buckthorn, sesame, tomato, pomegranate), cereals (amaranth, oat, rice bran, wheat germ, wheat bran), fruits and vegetables (buriti fruit, carrot, cloudberry, hiprose, olive husk, tomato) (Oreopoulou and Tzia 2007; Temelli et al. 2008).

Specialty oils obtained from food industry by-products of plant origin using $SCCO_2$ extraction and the studied range of extraction parameters are listed in Table 9.1. They mainly include seed or kernel (apricot and sour cherry kernels and grape, passion fruit, peach, pomegranate, pumpkin, sweet cherry seeds) cereal (rice bran and wheat germ) and vegetable (olive husk) oils.

The extraction and processing of fish oil using $SCCO_2$ have been very well established and PUFA concentrates are generally regarded as specialty lipids (Catchpole et al. 2009b). However, extraction of oils, which are rich in EPA and DHA from seafood processing by-products, is covered in this section and the references to the research available are listed in Table 9.1.

9.3.1.1 Pretreatment of By-Products

In general, supercritical processing of solid by-products needs essential pretreatments as drying and size reduction. Particle size and structure of the sample are major parameters during extraction; therefore, their influence on extraction is explained in detail in Sect. 9.3.1.2. Other pretreatments such as enzymatic

Table 9.1 Extraction of specialty oils from food industry by-products using $SCCO_2$

By-product	Bioactive compound	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Ref.
Fruit, vegetable and grain processing by-products										
Apricot kernel	Oleic acid, linoleic acid	5	<0.425–1.5	3.9	30–60	40–70	1–5 g/min		0–3 wt% ethanol	Özkal et al. (2005a)
Apricot kernel	Oleic acid, linoleic acid	5	<0.850	3.9	30–45	40–60	2–4 g/min	ni	0–3 wt% ethanol	Özkal et al. (2005b)
Grape seed	Oleic acid, linoleic acid, α-tocopherol, polyphenols	100	20–40 mesh	ni	25	80	69 g/min	60	–	Agostini et al. (2012)
Grape seed	Oleic acid, Linoleic acid	280 4,677	0.78	12	35	40	8.22 kg/kg[a]	300–450 min	–	Prado et al. (2012)
Grape seed	Oleic acid, linoleic acid, antioxidants	70	0.75	ni	18–22	40–50	1.7×10^{-4} kg/s	ni	–	Passos et al. (2010)
Grape seed	Oleic acid, linoleic acid	70	0.75	ni	16–20	40	1.7×10^{-4} kg/s	ni	–	Passos et al. (2009)
Grape seed	α-tocopherol	3	0.363–1.125	ni	25	40–80	25–95 kg/kg[a]	7 h	–	Bravi et al. (2007)
Grape seed	Oleic acid, linoleic acid	4	10–60 mesh	ni	20–30	35–45	0.4 mL/min	1 h S,[b] 1 h D	–	Cao and Ito (2003)
Grape seed	Oleic acid, linoleic acid	40	0.35–2.83	0.3–6.3	5–35	14–60	0.5–2 L/min at STP	3–5 h	–	Gómez et al. (1996)
Passion fruit seed	Oleic acid Linoleic acid	15	0.778	0.02	15–25	40–50	1.5–3 cm^3/min	200 min	–	Cardoso de Oliveira et al. (2013)
Peach seed	Oleic acid, linoleic acid	2–10	0.250–0.350	ni	15–19.8	40–51	0.68–2.37 g/min	ni	2.5–5 mol % ethanol	Sánchez-Vicente et al. (2009)

(continued)

Table 9.1 (continued)

By-product	Bioactive compound	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Ref.
Pomegranate seed	Punicic acid, tocopherol	250	0.3–0.9	4.1	13.2–46.8	33.2–66.8	6.6–23.4 L/h	2 h	–	Liu et al. (2009)
Pumpkin seed	Phytosterols	66	0.25	9.2	40	40	0.44 kg/h 30 kg/kg[a]	4 h	–	Hrabovski et al. (2012)
Pumpkin seed	Oleic acid, linoleic acid, linolenic acid	3.25	0.250–2.360	9.2	20–50	40–70	0.06–0.30 L/h	100 min–5 h	0–10 vol. % ethanol, hexane	Salgın and Korkmaz (2011)
Pumpkin seed	Oleic acid, linoleic acid, linolenic acid	25	0.5	2	15–35	35–75	0.25 L/min	30–150 min	–	Mitra et al. (2009)
Pumpkin seed	Oleic acid, linoleic acid, linolenic acid	20	0.36	ni	18–20	35–45	0.05–0.082 cm/s[c]	ni	–	Bernardo-Gil and Cardoso Lopes (2004)
Pumpkin seed	Oleic acid, linoleic acid, linolenic acid	ni	60–80 mesh	6.11–6.25	25–30	45	30–40 kg/h	2 h	–	Wenli et al. (2004)
Sour cherry kernel	Oleic acid, linoleic acid, tocopherols, β-carotene, polyphenols	100	ni	3.91	30	60	25 g/min	2 h	0–3 % ethanol	Yılmaz and Gökmen (2013)
Sweet cherry seed	Oleic acid, linoleic acid, phytosterols	ni	<1.25	10.88	18–22	40–60	0.02–0.08 cm/s[c]	60–150	–	Bernado-Gil et al. (2001)
Olive husk oil	Tocopherols, carotenoids, chlorohylls	25	0.33	6	25–35	40–60	1 L/min	ni	5 % ethanol	Gracia et al. (2011)
Rice bran oil	γ-oryzanols	10–35	ni	<10	25–35	40–60	6–14 g/min 40–120 kg/kg[a]	ni	–	Wang et al. (2008)
Rice bran oil	Oleic acid, linoleic acid	40	ni	8	20–35	45–85	25 g/min	30 min	–	Sparks et al. (2006)

Rice bran oil	Tocopherols, tocotrienols	ni	0.21	ni	15–25	25–60	0.0756 kg/h	8 h	–	Sarmento et al. (2006)
Rice bran oil	Oleic acid, linoleic acid	20	ni	ni	34.5–68.9	40–80	1.65 g/min	1–4 h	–	Perretti et al. (2003)
Rice bran oil	Oleic acid, linoleic acid, squalene	30	ni	ni	20.7–41.4	40–70	ni	8 h	–	Kim et al. (1999)
Rice bran oil	Phytosterols	5	>0.297	6	48.3–62	70–100	ni	90 min	–	Kuk and Down (1998)
Wheat germ	Tocopherols, polyphenols	2	0.75	<1	14.8–60.2	40–60	2 g/min	10–60 min	–	Gelmez et al. (2009)
Wheat germ	Tocopherols	220, 450	ni	ni	20–40	40–60	6–8 kg/h	2–4 h	–	Zacchi et al. (2006)
Wheat germ	Tocopherols, tocotrienols carotenoids	ni	0.35–0.50	ni	25–38	55	1.5 L/min	3 h	–	Panfili et al. (2003)
Wheat germ	Tocopherols	5	20–100 mesh	4.3–11.5	13.8–41.4	35–50	1–3 mL/min	120 min	–	Ge et al. (2002a)
Wheat germ	Tocopherols	ni	0.505	5.1	13.8–41.4	30–50	1.5–2.5 mL/min	90 min	–	Ge et al. (2002b)
Wheat germ	Oleic acid, linoleic acid, linolenic acid, tocopherols	25	0.75	ni	5–30	10–60	0.5–2 L/min at STP	3 h	–	Gómez and de la Ossa (2000)
Seafood processing by-products										
Brazilian redspotted shrimp waste	PUFA, astaxanthin	7	0.331	5.5	20–40	40–60	4.17×10^{-5} kg/s 1.5 L/min	20 min S,[b] 200 min D	–	Sánchez-Camargo et al. (2011a)
Brazilian redspotted shrimp waste	PUFA, astaxanthin	7	0.331	5.5	30	50	8.33×10^{-5} kg/s	20 min S,[b] ni D	10 % ethnol	Sánchez-Camargo et al. (2011b)

(continued)

Table 9.1 (continued)

By-product	Bioactive compound	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Ref.
Brazilian redspotted shrimp waste	PUFA, astaxantine	7	0.331	ni	30	50	3 L/min at STP	100 min	5–10 wt% ethanol	Sánchez-Camargo et al. (2012)
Fish by-products[d]	PUFA	100	1–10	ni	25	40	10 kg/h	3 h	–	Rubio-Rodríguez et al. (2012)
Hake by-products	PUFA	100	1–10	8.4–51.5	10–57.7	40	5–20 kg/h	3 h	–	Rubio-Rodríguez et al. (2008)
Longtail tuna heads	PUFA	2	0.2–0.5	ni	13.2–46.8	38.2–71.8	0.3–3.7 mL/min	1.8–16 h	20 vol.% ethanol	Ferdosh et al. (2013)
Northern shrimp by-products	PUFA	10	2	ni	15–35	50–40	3–5 L/min	90 min	–	Amiguet et al. (2012)
Trout by-products (Heads, spines, viscera)	PUFA	35 g[e]	< 2[e]	1.5, 7.9, 0.2[f]	50	60	10 g/min	ni	–	Fiori et al. (2012)

[a]Solvent to feed ratio
[b]*S* static extraction, *D* dynamic extraction
[c]Superficial velocity
[d]Off cuts from hake, orange roughy and salmon and livers from jumbo squid
[e]Heads and spines
[f]Moisture contents for spines, heads and viscera, respectively, ni not indicated

treatment of cell walls, roasting in case of extraction of specialty oils, defatting in case of extraction of polyphenols (see Sect. 9.3.4.2) are common. In case of fractionation of liquid by-products to enrich bioactive compounds, modification of the composition of the raw material is required. These pretreatments include esterification of free fatty acids (FFAs) and methanolysis of glycerides to form fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs) in the oil by-products (see Sect. 9.3.2.2).

Moisture has a great impact on oil extraction. The effect might be positive as expanding the solid matrix allows an increased permeability of solute and solvent; furthermore it can act as a cosolvent for polar compounds. On the other hand, the effect of moisture might be negative because of impeding the diffusion of oil or bioactive compounds.

It was demonstrated that the oil yield was not affected significantly by the moisture content (0.3–6.3 %) of the grape seeds (Gómez et al. 1996). In wheat germ extraction, the tocopherol yield increased with a decrease in moisture content from (11.5 to 8.2 and 5.1) % but decreased with a further reduction to 4.3 % indicating a shrinkage of the germ particles (Ge et al. 2002b). In case of carotenoids, it was reported that water acts as a cosolvent for the extraction of polar lutein, whereas presence of water is not favorable for the relatively nonpolar lycopene and carotenes.

Fish processing by-products need to be freeze-dried in order to reduce their moisture to values below 20 %. The oil yield from hake by products (1–10 mm) at 25 MPa and 40 °C using 250 kg CO_2/kg by-product, increased remarkably from (16 to 24) kg/kg when moisture decreased from (51.1 to 17.8) % but there was not a significant difference in oil yield when moisture further decreased from (17.8 to 8.4) % (Rubio-Rodríguez et al. 2008).

For the extraction of oil from seeds a pretreatment of the cell walls is preferred to grinding. The pretreatment of grape seeds has been performed with a cell wall degrading enzyme cocktail containing cellulose, protease, xylanase and pectinase in order to enlarge the broken/intact cell ratio, thus increasing oil availability. The maximum obtained yield was reported to be 16.5 % which is about 44 % higher than the 11.5 % yield obtained with untreated seeds. The pretreatment yielded a linear fast extraction period and an asymptotic slow extraction period which contributed to the oil recovery only by (3–8) % (Passos et al. 2009), parallel to the effect of grinding on extraction rate.

Roasting is required to maintain the stability of unsaturated fatty acids. Roasting forms various neo-formed compounds having antioxidant activity. In addition, it may increase the extractability of several naturally occurring antioxidants by modifying the food matrix. In the meantime, some heat labile antioxidant compounds are lost during extended heat treatment. From the viewpoint of total antioxidant capacity of oil there is a balance between neo-formed compounds and degradation of naturally occurring antioxidants (Yılmaz and Gökmen 2013).

Roasting not only improves the stability of wheat germ by preventing the autoxidation of unsaturated fatty acids which leads to off-flavors but also enhances the antioxidant activity (Krings et al. 2000; Krings and Berger 2001) and generates

DNA-protective properties (Krings et al. 2006). The increased antioxidant activity of ethanolic extracts of wheat germ roasted at elevated temperatures (160–200 °C) compared to fresh wheat germ has been attributed to Maillard-type antioxidants produced during roasting (Krings et al. 2000). As an alternative, wheat germ enzymes were inactivated using far-infrared rays for 8 min at 105 °C (Ge et al. 2002a, 2002b).

Roasting cherry kernels at 160 °C for 30 min did not affect the fatty acid composition of the cherry kernel oil, increased total phenolic content (4.5 times) but decreased total tocopherols (9.8 %) and caused formation of hydroxyl methylfurfural (1.4 mg/L) in resulting oil (Yılmaz and Gökmen 2013). It was reported that there was no differences in fatty acid composition of rice germ oils prepared at different roasting temperatures and times.

9.3.1.2 Extraction Parameters

Particle size and structure of the matrix, pressure and temperature of the extraction, solvent flow rate, extraction time and cosolvent concentration are extraction parameters that affect oil yield and the bioactive composition in oil.

Effects of Particle Size and Structure. Vegetable oil is deposited in the oil cells of the vegetable matrix and protected by cell walls. Grinding is necessary to release the oil from intact oil cells. Some of the cells are broken up during grinding and some part of the oil is released from the cells and directly proposed to the solvent on the surface of the particles. The rest remains unreleased in the intact cells. As a result, extraction occurs in two periods as fast and slow extraction periods. The released oil is extracted in the fast extraction period with a rate controlled by its diffusion and convection in the solvent. When the released oil is removed, the unreleased oil in the intact cells is extracted during the slow extraction period with a rate controlled by the diffusion of the oil from the interior of the particles to the surface (Özkal et al. 2005a).

Özkal et al. (2005a) carried out a systematic study about the effect of particle size on extraction yield of apricot kernel oil. Four different sized particles with mean diameter ($<$0.425, $<$0.85, 0.92 and 1.5) mm were investigated. It was demonstrated that grinding of apricot kernel before extraction not only increased the interfacial area but also released oil from the broken cells. The apricot kernel sample with particle diameter $<$0.425 mm contained the released oil mostly. However, in the sample with particle diameter of 1.5 mm, about 32 % of the oil was released and the rest was unreleased oil. Consequently, the duration of the fast extraction period, where the released oil was extracted, was shorter for the sample with particle diameter of 1.5 mm (25 min) compared to the duration of the fast extraction period for the sample with particle diameter $<$0.425 mm (61 min).

The released oil was completely extracted at the end of the fast extraction period and the unreleased oil was extracted during the slow extraction period. Since the extraction is controlled by the diffusion of the oil inside the particles the extraction rate was slow and oil recovery was much smaller compared to that in the fast

extraction period. Considering the oil recovered during 90 min of extraction, oil recovered in the slow extraction period was only (3–7) % for the large particles (particle diameter 0.92 and 1.5 mm). However, insignificant amount of oil was recovered in the slow extraction period when the particles were small (particle diameter <0.425 and <0.85 mm). Almost all initial oil in the apricot kernel sample with particle size <0.425 mm was recovered at the end of the fast extraction period. However, only 39 % of the oil in the sample with particle size of 1.5 mm was recovered during 90 min of extraction. Similar results were reported during $SCCO_2$ extraction of pumpkin seed oil (Salgın and Korkmaz 2011).

Results indicated that extraction rate is high if the oil is released on the surface of particles and that it is comparably very slow if it is embedded in the particles. The reduction of particle size of the vegetable matrix is required in order to decrease the extraction time; otherwise extraction in the slow extraction period may not be feasible. On the other hand, the production of very small particles can largely increase grinding cost and might produce bed caking with formation of channels along the bed in which supercritical fluid can preferentially flow, thus reducing the extraction efficiency.

Oil composition and structure of the fish by-products play an important role in extraction of oil from seafood processing by-products. At the beginning of extractions, the process is controlled by the oil solubility in $SCCO_2$, i.e. the internal mass transfer is constant. The solubility of fish oils rich in triglycerides as salmon oil are lower than those rich in wax esters as orange roughy oil. The internal mass transfer resistance which is controlled by diffusion of oil in solid can be considered negligible for fish by-products with mostly extracellular oil as is the case of orange roughy oil or oil weakly bound to the protein matrix, as observed for salmon oil. At 25 MPa and 40 °C, initial slopes of extraction curves of oils from orange roughy and salmon offcuts are higher than those of hake offcuts and jumbo squid liver although salmon and hake offcuts and jumbo squid liver oils are all rich in triglycerides (Rubio-Rodríguez et al. 2012).

The internal mass transfer resistance is reduced by decreasing the particle size of fish by-products as in the case of seeds and kernels. In order to study the influence of the internal mass transfer on the extraction yield, Rubio-Rodríguez et al. (2008) screened the freeze-dried hake offcuts with a 5 mm sieve. Particles smaller than 5 mm resulted to be mostly hake muscle with an average total fat content of 20.7 kg oil/100 kg protein while particles larger than 5 mm were mostly skin with an average total oil content of 27.7 kg oil/100 kg protein. The initial oil extraction rate at (25 and 50) MPa, and 40 °C was larger in hake muscle than in hake skin. The comparison of the slope of the extraction curves obtained at 40 °C for hake muscle at zero time (6.8 g oil/kg CO_2 at 25 MPa and 10.9 g oil/kg CO_2 at 50 MPa) were closer to the reported fish oil solubility (6.9 g oil/kg CO_2 at 25 MPa and 15.8 g oil/kg CO_2 at 50 MPa as calculated from the Chrastil correlation) than the slope of the extraction curves obtained for hake skin (4.7 g oil/kg CO_2 at 25 MPa and 8.8 g oil/kg CO_2 at 50 MPa) indicating that internal mass transport is slower in hake skin than in hake muscle.

Effect of Pressure. Extraction pressure is a dominant parameter that affects the oil yield. Elevating pressure increases the solubility of specialty oils in $SCCO_2$. Increase in the solubility of the oil in $SCCO_2$ increases the driving force in the fluid phase and consequently extraction rate in the fast extraction period. On the other hand, the diffusivity of oils in $SCCO_2$ decreases at high pressures. Consequently, mass transfer resistance increases, resulting in a decrease in the extraction rate. However, the increase in the driving force affects the extraction rate more than the increase in mass transfer resistance, and then the duration of the fast extraction period decreases as pressure increased.

The apricot kernel (particle diameter <0.852 mm) oil yield obtained at the end of the fast extraction period was constant (0.413 g/g kernel) but the extraction rate increased with pressure. At constant temperature of 50 °C and $SCCO_2$ flow rate of 3 g/min, increase in pressure from (30 to 60) MPa caused a 2.8 fold decrease in the duration of the fast extraction period (Özkal et al. 2005a).

Extraction pressure was reported as the dominant parameter to affect the grape seed (Passos et al. 2010), pomegranate seed (Liu et al. 2009), rice bran (Sparks et al. 2006), wheat germ (Gelmez et al. 2009) oil yield. Although the optimum pressure is dependent on other extraction parameters, the optimum extraction pressure for specialty oils from fruit, vegetable and grain processing by-products can be generalized to be between (35 and 50) MPa. The optimum extraction pressure was reported to be 50 MPa for pumpkin seed oil (Salgın and Korkmaz 2011), 45 MPa for apricot kernel oil (Özkal et al. 2005b), 44 MPa for wheat germ oil (Gelmez et al. 2009), 35 MPa for rice bran oil (Sparks et al. 2006).

The triglyceride and fatty acid profiles are roughly unaffected by pressure (Passos et al. 2010). The bioactive yield in the specialty oils increase with pressure but bioactive concentration decreases because of the diluting effect of oil. The tocopherol and phenolic concentration in wheat germ oil decreased with pressure especially during short extraction times (Gelmez et al. 2009). At constant temperature of 40 °C increasing pressure from (25 to 35) MPa increased olive husk oil yield, carotenoids and chlorophyll concentration but decreased tocopherol concentration in the oil (Gracia et al. 2011). The highest astaxantine concentration (1,074 μg/g extract) in the oil extracted from Brazilian redspotted shrimp waste (including head, tails and shell; 0.331 mm) was obtained at highest pressures (37 MPa) and lowest temperatures (43 °C) (Sánchez-Camargo et al. 2011a).

The yield of lipids from marine sources is lower than that of vegetable sources. Pressure was not a parameter of significant influence on oil yield but EPA and DHA concentrations increased with pressure (Sánchez-Camargo et al. 2011a). Maximum oil yield of $SCCO_2$ extraction from freeze-dried redspotted shrimp waste varied between (1.7–2.2) % for pressure range between (20–40) MPa. At a constant temperature of 40°C and a constant flow rate of 10 kg/h, increasing pressure from (10 to 25) MPa increased the initial extraction rate of oil from hake offcuts (1–10 mm) significantly, whereas the increase in the extraction rate was not very significant between (25 and 57.7) MPa (Rubio-Rodríguez et al. 2008). Similarly, at constant temperature (55 °C) and flow rate (2 mL/min), the total oil yield from longtail tuna heads increased from (3.5 to 35.3) % when pressure increased from

(13.2 to 46.8) MPa (Ferdosh et al. 2013). During the extraction of oil from Northern shrimp waste, at 15 MPa and 50 °C a yield of 11 mg of oil/g dried by-products was reached in 20 min with total fatty acid content of 620 mg/g of oil. The extension of extraction time did not change the yield. The oil yield at 35 MPa, 40 °C was reached in 90 min as 137 mg of oil/g dried by-product. Total fatty acid content of this oil was 795 mg/g oil including 78 mg/g EPA and 79 mg/g DHA (Amiguet et al. 2012).

Effect of Temperature. At low pressures the solubility of oils in $SCCO_2$ decreases with the rise of temperature due to the reduced density of CO_2 at higher temperatures. However, at high pressures the solubility of oils in $SCCO_2$ increases with increase in temperature due to the increase in their volatility. The solubility behavior is reversed after the crossover pressure. Some reported crossover pressures were 20 MPa for peach seed oil (Sánchez-Vicente et al. 2009), (20–25) MPa for wheat germ oil (Gómez and de la Ossa 2000); 26.2 MPa for vitamin E (Ge et al. 2002a, 2002b); (20–30) MPa for apricot kernel (Özkal et al. 2006) and pumpkin seed (Salgın and Korkmaz 2011) oils; 32 MPa for pomegranate seed oil (Liu et al. 2009) and (35–42) MPa for rice bran oil (Perretti et al. 2003).

Above the crossover pressure, the increase in the solubility of oil in $SCCO_2$ increases the driving force in the fluid phase. The mass transfer resistance decreases as a result of increase in diffusivity of oil in $SCCO_2$. Combined with the increase in driving force, the overall effect of temperature is an increase in extraction rate in the fast extraction period. Similarly, the effective diffusivity of oil in the solid particles increases with temperature. However, this increase is not significant and does not affect the yield in the slow extraction period. In the literature, optimum extraction temperatures, were reported to be above the crossover pressures and they ranged between (40 and 70) °C. Between (40 and 45) MPa, the optimum extraction temperature was reported to be 60 °C for apricot kernel oil (Özkal et al. 2005b) and 50 °C for pomegranate seed oil (Liu et al. 2009).

The time required to reach the apricot kernel (<0.852 mm) oil yield of 0.413 g/g at the end of the fast extraction period decreased as temperature increased. The decrease was about two fold when the temperature increased from (40 to 70) °C at 45 MPa and solvent flow rate of 3 g/min (Özkal et al. 2005a). However, temperature did not have a significant effect on wheat germ oil yield. Therefore, TPC and total tocopherol content (TTC) in wheat germ oil increased with temperature (Gelmez et al. 2009).

Crossover pressure of 30 MPa was reported for oil from longtail tuna head, oil yield increased with pressure and temperature above this pressure. At 40 MPa and 3 mL/min flow rate, the oil yield increased from (34.2 to 35.6) % with increasing temperature from (45 to 65) °C, respectively (Fiori et al. 2012).

Effect of Solvent Flow Rate. The oil yield increases due to the decrease in mass transfer resistance, as a result of increase in convection. During the extraction of apricot kernel oil (<0.852 mm), the duration of the fast extraction period decreased from (158 to 35) minutes as the solvent flow rate increased from (1 to 5) g/min at 45 MPa and 50 °C. At the end of the fast extraction period, the released oil (85.4 %) was recovered resulting in oil yield of 0.413 g/g kernel (Özkal et al. 2005a). The solvent flow rate exhibited a positive and significant effect on the pomegranate seed

oil yield (Liu et al. 2009). Prado et al. (2012) suggested a solvent to feed ratio of 8.22 for the scale up of grape seed oil extraction but reported a ratio of 6.6 at 35 MPa and 40 °C for 240 min of extraction as the one which produced the best relationship between yield and cost.

An increase in the oil yield from longtail tuna heads was observed with increasing flow rate at constant temperature and pressure. At 40 MPa and 40 °C oil yield increased from (22.1 to 34.2) % when flow rate increased from (1 to 3) mL/min (Fiori et al. 2012). On the other hand, at 25 MPa and 40 °C, the extraction rate from hake by-products increased significantly when the solvent flow rate increased from (5 to 10) kg/h, but decreased when the solvent rate increased from (10 to 20) kg/h (Rubio-Rodríguez et al. 2008).

Effect of Cosolvents. Addition of polar cosolvents as ethanol increases the solvating power of $SCCO_2$ by increasing its polarity. Although there have been some research on the effect of cosolvents on the extraction yield, usually it is not essential for the extraction of specialty oils. Addition of 3 wt% ethanol to $SCCO_2$ increased the solubility of apricot kernel oil 1.5 times compared to its solubility in pure $SCCO_2$, resulting in an increase in the driving force. During the extraction of apricot kernel (particle diameter <0.852 mm) oil, the duration of the fast extraction period at 45 MPa and 50 °C with 3 g/min solvent flow rate, deceased from (55 to 35) minutes (Özkal et al. 2005a).

The addition of ethanol to $SCCO_2$ conferred a significant improvement on the extraction yield of lipids from redspotted shrimp waste at 30 MPa and 50 °C, and also increased the proportion of ω-3 fatty acids in the extracts. An increase of 136 % in the total lipid extraction yield was observed when the proportion of ethanol was increased from (5 to 15) wt%. Maximum recoveries of 93.8 % for lipids, with regard to the initial content of waste, occurred with 15 wt% of ethanol, as well. The best results for the recovery of EPA and DHA were also obtained under these conditions (Sánchez-Camargo et al. 2012).

Effect of Extraction Time. Time has a significant effect on extraction yield of specialty oils and on the bioactive concentration in oils. Wheat germ oil yield increased with time especially at low pressures up to about 45 min. The effects of pressure and time on extraction yield dominate the changes in TPC and TTC of the wheat germ extracts. For short extraction times (35–40 min), TTC of the extracts decreased with pressure up to (35–40) MPa; however, for long extraction times (>40 min) and at high pressures (>45 MPa), TPC and TTC of the extracts increased with pressure (Gelmez et al. 2009). Zacchi et al. (2006) reported a decrease in α- and β-tocopherol concentration in wheat germ oil with time at 20 and 40 MPa and at 40 °C. A crossover time of 45 min was reported at 28 MPa and at 55 °C with 2 mL/min solvent flow rate where vitamin E yield from wheat germ decreased with temperature below this time and increased with temperature above it (Ge et al. 2002b). During the wheat germ extraction, maximum tocopherol and tocotrienol concentration was obtained between (50 and 100) min and carotenoids concentrated after 150 min (Panfili et al. 2003).

9.3.1.3 Optimization of Extraction Parameters

Table 9.2 summarizes the optimum parameters for maximum oil and bioactive yields, and product characteristics of the wheat germ oil obtained at optimized extraction conditions. The results show that $SCCO_2$ extraction is an important tool to obtain extracts with different antioxidant (phenolics and tocopherols) concentrations and activities by changing the extraction conditions namely pressure, temperature and extraction time. The extract is basically wheat germ oil (in which minor components such as phenolics and tocopherols are also present in dissolved state) 91 % of which was recovered by using $SCCO_2$ at 44.2 MPa and 40 °C for 48 min. However, the selectivity of the extraction process for these minor components was reflected in the TPC, TTC and AA of the extracts which were high at extraction conditions where the extraction yield was low (14.8–16.5 MPa, 40–60 °C and 10 min). These conditions should be preferred when the purity of the extracts in terms of antioxidants (phenolics and tocopherols) are more important than the yields. When the total or individual yields are more important, extraction conditions should be around (36–46) MPa, (40–60) °C, and 60 min; the only exception is tocopherol for which the maximum yield occurs at 10 min. The overall optimum set of conditions found by maximizing all the dependent variables together, were calculated to be 33.6 MPa, 58 °C and 10 min. Tocopherol yield was 0.33 mg tocopherol/g germ under these conditions, corresponding to almost 100 % recovery (Gelmez et al. 2009).

On the other hand, temperature (40–60 °C) and pressure (20–40 MPa) had significant effects on astaxanthin extraction yield. The largest amount of extract (20.7 μ g/g waste) was obtained at 37 MPa and 43 °C with 39 % recovery. The highest concentration of astaxanthin in the extracts was found at the same conditions (Sánchez-Camargo et al. 2011a).

9.3.1.4 Characterization of Products

Tables 9.3 and 9.4 show the fatty acid contents of specialty oils obtained from fruit, vegetable and grain processing, and seafood processing by-products, by $SCCO_2$ extraction, respectively. The oils obtained from fruit, vegetable and grain processing by-products are rich in unsaturated fatty acids (UFAs). Pomegranate seed oil is rich in punicic acid (60 %). Wheat germ oil has a low ω-6 to ω-3 fatty acid ratio (Table 9.3).

The oils obtained from seafood processing by-products are rich in long chain PUFAs, especially with a very low ω-6 to ω-3 fatty acid ratio (Table 9.4). Lipid profiles of oils extracted from trout heads, spines and viscera at 50 MPa and 60 °C were reported by Fiori et al. (2012). The amount of UFA was within the range of (72.6–75.3) % in oil oils. EPA and DHA content in oil from spines, heads and viscera were 8.7 % and 7.3 %, 7.9 % and 6.3 %, and 6.4 % and 6.0 %, respectively. Lipids, containing ω-1 PUFA chains were observed with an amount of 3 % in all the

Table 9.2 Effects of extraction parameters on oil yield, bioactive yield and bioactive composition in wheat germ oil extracted by $SCCO_2$ (2 g sample, 2 g/min $SCCO_2$ flow rate, Gelmez et al. 2009)

		P (MPa)	*T* (° C)	Time (min)
Maximum oil yield (%)	9	44.2	40	48
Maximum phenolic yield (mg GAE/g germ)	0.44	46.0	60	60
Maximum tocopherol yield (mg/g germ)	0.35	40.5	60	10
Maximum antioxidant yield (mg $DPPH^{\bullet}$/g germ)	3.57	36.1	60	60
Maximum TPC (mg GAE/g extract)	9.55	14.8	59	10
Maximum TTC (mg /g extract)	9.62	16.4	40	10
Maximum AA (mg $DPPH^{\bullet}$/g extract)	86	14.8	48	10
Optimum extraction conditions		33.6	58	10
Oil yield (%)	5.3			
TPC (mg GAE/g extract)	6.7			
TTC (mg/g extract)	6.7			
AA (mg $DPPH^{\bullet}$/g extract)	57.3			

oils. Oils from heads and spines were essentially composed of TAG (98 %). In viscera oil the molar distribution ratio of TAG:DAG:FFA was 87:8:5.

Tocol and phytosterol contents of the oils extracted from fruit, vegetable and grain processing by products are given in Tables 9.5 and 9.6, respectively. Unlike most oils, pumpkin seed oil contains more Δ-7- and Δ-5- sterols (Table 9.6).

9.3.2 Fractionation of Bioactive Lipid Components

Fractionation of bioactive lipid components can be classified as concentration of PUFAs and enrichment of minor lipid components. PUFAs are concentrated generally from fish oils. Minor lipid components (tocopherols, phytosterols, squalene, and carotenoids) are enriched either from specialty oils or from oil by-products, mainly from oil DOD. $SCCO_2$ processing of fish oils is a broad area where the commercially produced fish oils for human consumption are further processed for PUFA concentration (Eltringham and Catchpole 2008; Catchpole et al. 2009b). Therefore, the discussion in this chapter will be devoted only to the enrichment of PUFAs in oils extracted from seafood processing by-products using either $SCCO_2$ or an organic solvent.

9.3.2.1 Solubility of Lipids and Minor Lipid Components in $SCCO_2$

A large compilation of literature data and their correlation for solubility behavior of fatty acids, mono-, di-, and triglycerides, fatty acid esters in $SCCO_2$ can be found in Güçlü-Üstündağ and Temelli 2000 and for minor lipid components in Güçlü-Üstündağ and Temelli 2004.

Table 9.3 Fatty acid composition of specialty oils extracted by $SCCO_2$ from fruit, vegetable and grain processing by-products (%)

		Fatty acid	Apricot kernel[a]	Grape seed[b]	Peach seed[c]	Pomegranate Seed[d]	Pumpkin seed[e]	Sour cherry kernel[f]	Sweet cherry Seed[g]	Wheat germ[h]
C14:0		Myristic	–	0.11	0.05	–	–	–	–	0.09
C16:0		Palmitic	5.71	8.13	5.5	3.84	9.19	7.24	5.26	16.8
C16:1	ω-7	Palmitoleic	0.78	0.15	0.4	0.16	–	–	0.27	0.15
C18:0		Stearic	1.30	4.05	1.9	3.19	7.33	1.33	2.15	0.5
C18:1	ω-9	Oleic	67.37	15.10	73.0	8.81	31.64	44.99	32.64	13.6
C18:2	ω-6	Linoleic (LA)	24.84	71.20	18.2	11.85	47.26	41.81	40.84	59.7
C18:3	ω-3	Linolenic (ALA)	–	0.57	0.05		0.58	4.63	1.10	7.3
C18:3	ω-5	Punicic	–	–	–	60.96	–	–	10.11	–
C20:0		Arachidic	–	0.22	0.1	0.64	–	–	1.50	0.11
C20:0	ω-11	Gadoleic	–	0.07	–	0.66	–	–	1.21	1.45
		SFA (%)	7.01	12.4	7.5	7.67	16.52	8.57	8.91	17.41
		MUFA (%)	68.15	15.32	73.4	9.63	31.64	44.99	34.12	15.2
		PUFA (%)	24.84	71.77	18.25	72.81	47.84	46.44	52.05	67.0
		UFA (%)	93.00	87.09	91.65	82.44	79.48	91.43	86.17	82.2
		UFA/SFA	13.27	7.02	12.22	10.75	4.81	10.67	9.67	4.72
		ω-6/ω-3	–	124.91	364.00	–	81.48	9.03	37.13	8.18

[a]30 MPa, 50 °C, 3 g/min (Özkal et al., 2005b)
[b]35 MPa, 40 °C (Prado et al. 2012)
[c]19.8 MPa, 51 °C (Sánchez-Vicente et al. 2009)
[d]30 MPa, 50 °C, 1.5 L/h (Liu et al. 2009)
[e]30 MPa, (40–70) °C (Salgın and Korkmaz 2011)
[f] 30 MPa, 60 °C (Yılmaz and Gökmen 2013)
[g]18–22 MPa, (40 and 60) °C (Bernado-Gil et al. 2001)
[h]68 MPa, 80 °C (Eisenmenger and Dunford 2008)

Table 9.4 Fatty acid composition of fish oils extracted by $SCCO_2$ from seafood processing by-products (mg/g oil)

Fatty acid			H[a]	OR[a]	S[a]	JS[a]	NS[b]
C14:0		Myristic	19.00	4.00	40.40	39.00	43.21
C16:0		Palmitic	129.00	6.40	143.00	141.00	78.72
C16:1	ω-7	Palmitoleic	34.00	44.00	59.00	43.00	94.15
C18:0		Stearic	21.00	2.50	46.40	43.00	14.14
C18:1 *trans*	ω-9	Elaidic	–	–	–	–	23.81
C18:1	ω-9	Oleic	142.00	213.00	146.00	42.00	103.54
C18:1	ω-7	Vacenic	22.00	24.00	28.90	23.00	–
C18:2 *trans*	ω-6	Linoeladic	–	–	–	–	3.38
C18:2	ω-6	Linoleic (LA)	7.00	4.70	93.00	5.80	8.33
C18:3	ω-6	γ-Linolenic (GLA)	1.90	1.40	5.50	3.10	–
C18:3	ω-3	α-Linolenic (ALA)	2.60	–	14.00	2.90	5.08
C18:4	ω-3	Stearidonic	3.20	–	5.90	2.00	–
C20:1	ω-9	Gadoleic	37.00	50.00	13.10	24.00	85.51
C20:2	ω-6	Eicosadienoic	–	–	–	–	2.63
C20:3	ω-6	Dihomo-γ-linolenic	0.82	1.50	3.20	1.60	–
C20:4	ω-6	Arachidonic (AA)	5.50	–	6.70	12.700	2.08
C20:5	ω-3	Eicosapentanoic (EPA)	36.00	3.20	79.00	127.00	78.00
C22:1	ω-9	Euricic	4.20	6.50	–	1.50	59.90
C22:1	ω-11	Cetoleic	28.00	19.00	–	5.60	106.07
C22:4	ω-6	Adrenic	4.00	–	–	5.40	–
C22:5	ω-3	Decosapentaenoic (DPA)	8.00	–	38.40	22.00	6.53
C22:6	ω-3	Decosahexanoic (DHA)	82.00	5.20	63.00	130.00	79.66
C24:1	ω-9	Nervonic	7.80	2.90	2.50	2.80	3.02
		TOTAL	595.02	388.3	788.00	677.4	797.76
		SFA (%)	28.40	3.32	29.16	32.92	17.06
		MUFA (%)	46.22	92.56	31.66	20.95	59.67
		PUFA (%)	25.38	4.12	39.18	46.13	23.28
		ω-3 FA (%)	22.15	2.16	25.42	41.91	21.22
		ω-6 FA (%)	2.31	1.96	12.91	2.35	1.80
		ω-6/ω-3	0.10	0.90	0.51	0.06	0.08

[a]Offcuts from hake (H), orange roughy (OR), salmon (S), livers from jumbo squid (JS), 25 MPa, 40 °C (Rubio-Rodríguez et al. 2012)
[b]Nothern sphrimp waste (NS) 35 MPa, 40 °C (Amiguet et al. 2012)

In a homologues series, solubility in $SCCO_2$ decreases with increase in molecular weight and polarity of solutes. The effect of solute properties on solubility depends on operating conditions. An isothermal increase in the pressure, and a temperature increase at constant CO_2 density, lead to an increase in solubility of lipids and minor lipid components. Retrograde solubility behavior was observed for liquid solutes whereas the solid solutes were in the non-retrograde region between (35 to 100) °C and upto 36 MPa (Güçlü-Üstündağ and Temelli 2000).

Table 9.5 Tocol contents of specialty oils extracted by $SCCO_2$ from fruit, vegetable and grain processing by-products

Tocols (mg/100 g oil)	Peach seed[a]	Pomegranate seed[b]	Sour cherry kernel[c]	Rice bran oil[d]	Wheat germ[e]	Wheat germ[f]
α-tocopherol	–	5.18	89.47	179.18	4,800	1,336.2
β-tocopherol	–	–	183.80 (β + γ)	1.21	2,400	561 5
γ-tocopherol	440	277.41	–	54.67	0.0117	323.7
δ-tocopherol	–	13.14	38.88	–	0.0008	92.6
Total tocopherol	440	295.73	312.15	234.96	7,100	2,314
α-tocotrienol				96.16		
β-tocotrienol				0.71		
γ-tocotrienol				587.92		
δ-tocotrienol				19.37		
Total tocotrienol				704.16		
Total tocols				939.12		

[a]19.8 MPa, 40 °C (Sánchez-Vicente et al. 2009)
[b]30 MPa, 50 °C, 15 L/h (Liu et al. 2009)
[c]30 MPa, 60 °C (Yılmaz and Gökmen 2013)
[d]20 MPa, 40 °C, 8 h, 1.5 mL/min (Sarmento et al. 2006)
[e]24 MPa, 56 °C, 20 min (Gelmez et al. 2009)
[f]34.5 MPa, 43 °C, 90 min, 1.7 mL/min (Ge et al. 2002a)

Table 9.6 Phytosterol contents of specialty oils extracted by $SCCO_2$ from fruit, vegetable and grain processing by-products

Phytosterol (mg/100 g oil)	Pumpkin seed[a]	Sweet cherry seed[b]	Wheat germ[c]
Desmosterol	8.67	0.36	–
Colesterol	–	–	–
Campesterol	3.51	1.03	610
Campestenol	–	2.98	–
Sigmasterol	–	3.85	–
Stigmasterol	3.87	–	200
Chlesroterol	–	0.80	–
β-Sitosterol	10	83.41	294
Spinasterol	74.5	–	–
Δ5-Sterols	26.1	–	–
Δ7-Sterols	268	–	–
Total	294	–	3,750

[a]40 MPa, 40 °C (Hrabovski et al. 2012)
[b]mol %, (18–22) MPa, (40 and 60) °C (Bernado-Gil et al. 2001)
[c]68 MPa, 80 °C (Eisenmenger and Dunford 2008)

Squalene is the most soluble component among the minor lipid components. The solubility of α-tocopherol is very similar to that of squalene at low CO_2 densities. However, the available solubility data for α-tocopherol is very scattered.

Stigmasterol is less soluble than triolein, while β-carotene is the least soluble component in $SCCO_2$. The solubilities for γ-tocopherol and α-tocopherol are similar at 80 °C whereas crossover of isotherms are observed at (40 and 60) °C at pressures of (28 and 22) MPa, respectively, such that α-tocopherol solubility was higher at lower pressures whereas γ-tocopherol is more soluble at higher pressures (Güçlü-Üstündağ and Temelli 2004). The solubilities of oleic acid (which is a common fatty acid in oils of vegetable origin), its methyl ester, its triglyceride and minor lipid components in $SCCO_2$ in descending order can be given as methyl oleate, squalene, α-tocopherol, oleic acid, triolein, stigmasterol and β-carotene.

Although these findings are useful in providing information about the relative solubilities of fats and oils components in $SCCO_2$ as affected by solute properties, they are of limited use in the study of real mixtures. The differences in the effects of operating conditions on solubility and the presence of other components will affect the relative solubilities, therefore a study of multicomponent systems is required for optimal design of fractionation process (Güçlü-Üstündağ and Temelli 2004).

For the purpose of enrichment of tocopherols from oil DODs behaviors of binary systems α-tocopherol + CO_2 and methyl oleate + CO_2, ternary systems methyl oleate + tocopherol + CO_2 and a realistic system methyl esterified soybean oil DOD + CO_2 have been studied by Fang et al. (2008). The conclusions of their research indicate that both methyl oleate and α-tocopherol mole fractions in gas phase increase as pressure increases at constant temperature. Meanwhile, the CO_2 fraction in liquid phase rises with increasing pressure. The equilibrium concentration of methyl oleate is always much higher than the equilibrium concentration of α-tocopherol in CO_2. Moreover, the influence of temperature on gas composition is opposite to that of pressure. In the case of methyl oleate + CO_2 mixture the ranges for critical points are (13–14) MPa at 40 °C, (19–20) MPa at 60 °C and (23–24) MPa at 80 °C. In the case of α-tocopherol + CO_2, the distribution coefficient indicates that the components are poorly dissolved in each other. The distribution coefficients of α-tocopherol are one and two order of magnitudes lower than those of methyl oleate. Ternary phase behavior showed that with the increase of methyl oleate mass fraction in the feed, phase behavior tends to be close to that of the binary system of methyl oleate + CO_2. Low pressures and high temperatures lead to high selectivity, which is advantageous for separating methyl oleate from tocopherol with $SCCO_2$. In addition, at constant pressure and temperature, the separation factor increases as the initial tocopherol content decreases. For the realistic system at 40 °C the separation factor remains lower than 0.2 for all pressures lower than 15 MPa. As pressure increases the separation factor increases considerably, reaching 0.35 at 20 MPa. The increase of temperature offset as the effect of pressure to some extents. Critical pressure at 40 °C is approximately estimated in the pressure range from (27.8–29) MPa.

9.3.2.2 Pretreatment of By-Products

The concentration of long chain PUFA in the form of triglycerides has had limited success, because the fatty acids are more or less randomly distributed. The triglycerides must first be converted to methyl or ethyl esters or free fatty acids. Some concentration of esters or fatty acids is possible by direct fractionation using $SCCO_2$, but on the basis of chain length and not on the degree of unsaturation. Therefore, it is necessary to carry out either a pre-concentration step or a separation process that takes place in a supercritical environment, such as chromatography, urea fractionation or enzymatic fractionation (Eltringham and Catchpole 2008; Catchpole et al. 2009b).

DOD, a by-product of the edible oil refining process, is rich in bioactive compounds as tocopherols, sterols and squalene (Fang et al. 2008). To enrich the bioactives in DOD, modification of the composition of raw material, i.e. a process of pretreatment, is generally necessary. Pretreatment involves esterification of FFAs and methanolysis of glycerides to form FAMEs. The pretreatment can be performed chemically or enzymatically. In case of chemical modification of DOD, esterification is carried out with the catalysts sulfuric acid (H_2SO_4), hydrochloric acid (HCl) or Na. Methanolysis is carried out with catalysts sodium methoxide ($NaOCH_3$) and sodium hydroxide (NaOH) (Fang et al. 2008). Soybean oil DOD was modified by using H_2SO_4 and $NaOCH_3$ as catalysts of methyl esterification and methanolysis, respectively to form FAMEs (Fang et al. 2008). H_2SO_4 was used to form FAEEs in sunflower oil DOD (Vázquez et al. 2006). FAMEs in rice germ oil were formed using $NaOCH_3$ (Ko et al. 2012). Olive oil DOD was esterified in supercritical methanol (Akgün 2011). Two step enzymatic reaction was applied to modify soybean oil DOD by Torres et al. (2009). Sterol esterification with lipase from *Candida rugosa* and ethyl esterification with *Candida antartica* lipase (Novozyme 435) to obtain a product mainly comprised FAEEs, tocopherols and pytosterol esters together with minor amounts of squalene, FFAs, free sterols and triacylglycerols. To recover squalene from olive oil DOD, a by-product was obtained after distillation and ethylation of olive oil DOD. The raw material contained 52 wt% squalene, 22.7 wt% fatty acid esters and 1.9 % sterol type (Vázquez et al. 2007).

9.3.2.3 Processing Schemes

The process schemes for fractionation of bioactive compounds include $SCCO_2$ extraction, fractional extraction, fractional separation and column fractionation. These applications are classified and summarized in Table 9.7.

$SCCO_2$ Extraction. Tocols were efficiently enriched in rice germ oil by removal of methyl esters from the esterified rice germ oil (40 g) using $SCCO_2$. The enrichment of tocols was carried out at pressures between (12.4 and 15.8) MPa and at temperatures between (40 and 60) °C using a CO_2 flow rate of 5 L/min. The combination of 13.8 MPa and 60 °C was selected as the most suitable for the

Table 9.7 Fractionation of bioactive compounds from food industry by-products

By-product	Enriched bioactive compound	Pretreatment	Process scheme	Ref.
Enrichment of PUFA from seafood by-product oils				
Fish by-products	PUFAs	–	Fractional separation	Rubio-Rodríguez et al. 2012
Sardine by-products	EPA, DHA	–	Fractional extraction	Létisse and Comeau 2008
Enrichment of minor lipid components from specialty oils				
Corn bran oil	Phytosterols	–	Fractional extraction	Snyder et al. 1999
Corn fiber oil	Phytosterols	–	Fractional extraction	Snyder et al. 1999
Rice bran oil	Phytosterols	–	Fractional extraction	Snyder et al. 1999
Rice bran oil	α-tocopherol, phytosterols, oryzanol	–	Fractional extraction	Shen et al. 1996
Rice bran oil	Tocols	–	Fractional Separation	Sarmento et al. 2006
Rice bran oil	Tocopherol, phytosterols, oryzanol	–	Fractional Separation	Shen et al. 1996
Rice bran oil	Phytosterols, oryzanol	–	Column fractionation	Dunford et al. 2003
Rice bran oil	Phytosterols	–	Column fractionation	King and Dunford 2002
Rice bran oil	Phytosterols, oryzanol	–	Column fractionation	Dunford and King 2000
Rice germ oil	Tocols	Fatty acid ethyl esterification with $NaOCH_3$	$SCCO_2$ extraction	Ko et al. 2012
Wheat germ oil	Phytosterols		Column fractionation	Eisenmenger et al. 2006
Enrichment of minor lipid components from oil by-products				
Olive oil DOD	Squalene	Esterfication in supercritical methanol	$SCCO_2$ extraction	Akgün 2011
Olive oil DOD	Squalene	Distillation-ethylation	Column fractionation	Vázquez et al. 2007
Olive pomace	Tocopherols, phytosterols	–	Fractional Separation	Ibáñez et al. 2000
Palm oil FAD	Squalene	–	Column fractionation with thermal gradient	Al-Darmaki et al. 2012

(continued)

Table 9.7 (continued)

By-product	Enriched bioactive compound	Pretreatment	Process scheme	Ref.
Soybean oil DOD	Tocopherols, phytosterols, squalene	–	Fractional extraction	Chang et al. 2000
Soybean oil DOD	Phytosterol esters	Enzymatic esterification: Sterol esterification Enzymatic sterol and fatty acid ethyl esterification	Column fractionation	Torres et al. 2009
Soybean oil DOD	Tocopherol	Methyl esterification with H_2SO_4, methanolysis with $NaOCH_3$	Column fractionation	Fang et al. 2008
Soybean oil DOD	Phytosterols		Column fractionation	King and Dunford 2002
Sunflower oil DOD	Tocopherols, phytosterols	Chemical esterification: Fatty acid ethyl esterification with H_2SO_4	Column fractionation	Vázquez et al. 2006

efficient enrichment of tocols. The level of tocols (1,270 mg/100 g) in the residue obtained at these operating conditions was six times higher than the tocol level (192 mg/100 g) of the esterified rice germ oil. There were no significant differences in relative percentages of tocol homologues between esterified rice germ oil and the residue obtained by $SCCO_2$ extraction (Ko et al. 2012).

Olive oil DOD was esterified in supercritical methanol, and then squalene was extracted from the sample consisting of 66 % methyl esters using $SCCO_2$ at pressures between (8.82 and 12.18) MPa, temperatures between (41.6 and 58.4) °C and extraction times between (129.6 and 230.4) min. The highest squalene content at a fixed flow rate of 7 mL/min for 3 g sample, was obtained at 10.48 MPa, 52 °C and extraction time of 180 min. Squalene was concentrated in the raffinate upto 75 % (Akgün 2011).

Fractional Extraction. Fractional extraction refers to collecting fractions throughout the extraction period as a function of time where extraction temperature and/or pressure may be changed at certain intervals over time. This allows the collection of fractions with different compositions (Temelli 2009).

The fractional extraction of rice bran oil with extraction time was studied by Shen et al. (1996). 300 g of rice bran was extracted with dense $CO_2$with a flow rate of 2.5 kg/h, at temperatures (0–60) °C, and pressures (17–31) MPa over a period of 6 h. The extracted total oil, FFAs, α-tocopherol, sterols (campesterol, stigmasterol, β-sitesterol) and oryzanol components were measured at intervals. (40–64) % of FFAs was extracted in the first hour. About 90 % of α-tocopherol was recovered after 3 h at (40 and 60) °C, at 24 MPa. Approximately (4–14) % of oryzanol was extracted in the first hour during all runs and remained constant for the rest. Extraction curves of sterols were similar to that of the rice bran oil itself which is

mainly composed of triglycerides. Recovery of campesterol was incomplete. The earlier extracting components FFAs and α-tocopherol had larger apparent distribution coefficients whereas the late extracting component oryzanol had a smaller distribution coefficient.

Sterols from corn bran, corn fiber and rice bran oils were enriched by fractional extraction of lipid components (Snyder et al. 1999). Oil samples (0.3 g) were mixed with 0.15 g Hydromatrix (sorbent) and added into a 2.5 mL extraction vessel. The temperature was held constant at 80 °C during the course of fractional extraction. The extraction conditions in the four successive steps were 30 mL $SCCO_2$ at 55 MPa, 30 mL $SCCO_2$ + 10 % cosolvent at 13.8 MPa, 30 mL $SCCO_2$ + 10 % cosolvent at 27.6 MPa and 80 mL $SCCO_2$ + 10 % cosolvent at 41.4 MPa, respectively. Among the cosolvents used methyl tert-buthyl ether was found to be better than methanol for enrichment of sterols in all oils. However, the fractionation pattern for each oil was different. Sterol was enriched in the first, third and fourth fractions in case of rice bran, corn bran and corn fiber oils, respectively. The sterol concentration in corn fiber oil was increased from (13.2 to 166.2) mg/g during the fractionation process. In the case of corn bran oil, enrichment of sterol from (8.6 to 322) mg/g was achieved.

Létisse and Comeau (2008) applied a four step fractional extraction to hexane extracted oil (adsorbed on silica) from sardine by-products (head and tails) at (40 and 60) °C by changing the density of CO_2 gradually from (500 to 800) kg/m^3 to enrich EPA and DHA methyl esters. The pressures at each step were (9.1, 9.7, 11.5 and 16.4) MPa, respectively at 40 °C, and (12.9, 14.9, 18.7 and 26.4) MPa, respectively at 60 °C. Each step lasted for 45 min with $SCCO_2$ flow rate of 1 mL/min. The oil contained 9.15 % EPA and 9.46 % DHA initially. The concentration of saturated fatty acids in the oil fractions decreased from first to fourth step while EPA and DHA concentrations increased. In the fourth step, the oil fraction contained 4.28 % EPA and 7.53 % DHA at 40 °C, and 24.74 % EPA and 26.02 % DHA at 60 °C. Fractional extraction at 26.4 MPa and 60 °C (CO_2 density of 800 kg/m^3) with respect to time (15, 15, 15, 45 min in each step, respectively) yielded an oil fraction containing 94.50 % EPA and DHA methyl esters.

Fractional Separation. In fractional separation several separators are used in series. In this case, the extraction temperature and pressure are set to achieve as high a CO_2 density as possible so that the maximum amount of solutes can be extracted. Then, separator conditions are adjusted for decreasing CO_2 density such that fractions of, high, medium and low molecular weight corresponding to low, medium and high volatility compounds, respectively, are collected in sequence in the separators (Temelli 2009).

Sterols were enriched from olive pomace (300 g), first by performing a $SCCO_2$ extraction at pilot scale at 35 MPa, 50 °C with $SCCO_2$ and ethanol flow rates of 2,000 mL/h and 100 mL/h, respectively and subsequent fractionation by two successive depressurizations. Fractions obtained using high densities in the first separator (10–16.5 MPa, 40–60 °C) contained triglycerides, waxes and sterols. Enrichment of α-, β- and γ-tocopherols was achieved in the second separator (1 MPa, 25 °C) when working at low densities. The greatest concentration of

tocopherols was found when the pressure in the first separator was low (10–13.5 MPa) i.e. when the difference between the pressure of extraction cell and the separator was high. On the other hand, the effect of the separator temperature seemed to be less important (Ibáñez et al. 2000).

Enrichment of phytosterols from rice bran oil was achieved by Shen et al. (1996). Total extraction at 24.1 MPa and 40 °C was followed by a separation at either isothermal conditions at 40 °C between (8.6 and 11.2) MPa or isobaric conditions at 8.6 MPa between (40 and 50) °C. In all cases FFAs are concentrated in the extract. At isothermal conditions, oryzanol and tocopherol distributed in the raffinate while the phytosterols distributed in the extract.

Fractional separation of oils extracted from fish by-products (offcuts of hake and salmon and jumbo squid liver) were performed by Rubio-Rodríguez et al. (2012). Extraction performed at 25 MPa and 40 °C (CO_2 density of 800 kg/m^3) was followed by a two-step separation. The first separator was kept at 9 MPa and 35 °C (CO_2 density of 650 kg/m^3) and the second separator was kept at 5 MPa and 10 °C (CO_2 density of 468 kg/m^3). In this way, most of the triglycerides were recovered in the first separator and most of the fatty acids were recovered in the second separator. Higher amounts of oil were recovered in the first separator 63 %, 86 % and 83 % in hake, salmon and jumbo squid liver oil, respectively. The distribution of fatty acids varied significantly among different fish oils. In the case of hake and salmon oils, in which palmitic and oleic acids are the main fatty acids, the majority of the main acids were collected in the second separator. However, in the case of jumbo squid liver oil, in which palmitic acid and EPA are the most common fatty acids, the majority of the fatty acids were collected in the first separator.

Column Fractionation. A countercurrent column fractionation is used for separation of liquid feed mixtures. In general, a packed column has been used for this purpose with heaters to create a thermal gradient along the column height, leading to the formation of internal reflux to enhance separation efficiency. It is also possible to generate a reflux using an external reflux pump. Lab- and pilot scale columns (14.3–68 mm internal diameters and 0.6–13.6 m high) are operational in research facilities around the world (Temelli 2009).

Squalene in palm oil fatty acid distillate (FAD) was significantly enriched by means of supercritical fluid fractionation. The process showed higher sensitivity to pressure (10–20 MPa) and temperature (40–80 °C) compared to solvent to feed ratio (15–35), within the range studied. The optimum conditions at isothermal fractionation experiments were found to be at 18 MPa and 40 °C where squalene was concentrated from (2.2 to 8) % in palm oil FAD with a recovery of 50 %. Applying thermal gradient in supercritical fractionation has shown a significant increase in recovery of squalene, more than 95 % at pressures of 20 MPa, combined with temperature profile in four zones. From bottom to top, the first and second zones were at 45 °C, the third zone was at 60 °C, and the last zone was at 80 °C. The concentration of squalene which was 2 % in the feed was increased to 16 % in the top product. At constant pressure of 15 MPa, an increase of temperature reduced separation efficiency (ratio of squalene and FFA concentrations in top and bottom

streams) of squalene whereas increased separation efficiencies of FFA. This was explained by the fact that crossover pressures of oleic and stearic acids are lower than 15 MPa while for squalene it is above 25 MPa (Al-Darmaki et al. 2012).

Torres et al. (2009) performed extractions on pretreated soybean oil DOD in an isothermal counter current column (without reflux), with pressures ranging from (20 to 28) MPa, temperatures between (45 and 55) °C, and solvent to feed ratio 15 to 35 kg/kg. Using these extraction conditions, the FAEEs were completely extracted and thus the fractionation of tocopherols and phytosterol esters was studied. At 25 MPa, 55 °C with solvent to feed ratio of 35 the phytosterol esters were concentrated in the raffinate upto 82 wt% with a satisfactory yield of 72 %. The extract was separated in two separators. The first separator was maintained at (15–16) MPa at 55 °C while the second separator was maintained at 2 MPa and (15–12) °C. The tocopherols were concentrated in the first separator (40 %) and FAEEs were concentrated in the second separator (59 %). The computer aided optimization of the extraction process resulted in a raffinate product with 94.2 wt% of phytosterol ester purity and 80 % yield. The optimized extraction parameters were 30 MPa, 51 °C, solvent to feed ratio 42.7, reflux ratio (reflux/extract) 0.48 (Fornari et al. 2009).

Based on the phase equilibrium data that the separation factor between FAMEs and tocopherols changed markedly from (15 to 20) MPa, Fang et al. (2008) investigated initial pressures of (14, 16, 18) MPa in a packed column. The column temperature gradient was set from 40 °C at the bottom to 75 °C at the top, solvent to feed ratio 75. The optimum initial and final pressures were found to be (16 and 30) MPa, respectively, and a tocopherol fraction with 81 % purity was obtained.

Purification of squalene from pretreated olive oil DOD was performed in an isothermal counter current column without reflux at 70 °C and pressures ranging from (15–23) MPa, with a solvent to feed ratio of 13. Yield of squalene increased with pressure decrease both in the column and in the separator. The computer aided optimization of the extraction process resulted in raffinate product with 91 wt% squalene purity and high squalene recovery of 93 %. The optimized extractor pressure and temperature were 17.7 MPa and 70 °C, respectively. The separator pressure was 6.8 MPa, solvent to feed ratio was 51, and the reflux ratio (reflux/extract) was 3.6 (Vázquez et al. 2007).

Vázquez et al.(2006) performed the extraction of ethylated sunflower oil DOD in a pilot scale plant at 65 °C, with pressures ranging from (15 to 23) MPa and solvent to feed ratio from 15 to 30. The overall % recovery of the raffinate decreased with increased pressure but sterol ester, free sterol and tocopherol enrichment factors increased as the column pressure increased from (14 to20) MPa.

The fractionation of rice bran oil was carried out under isobaric and isothermal conditions over the range of (13.8–27.5) MPa, (45–80) °C, respectively. $SCCO_2$ and oil flow rates were (2 and 0.7) mL/min, respectively, as measured at ambient conditions. It was shown that fractionation at low pressure (13.8 MPa) and high temperature (80 °C) effectively removed FFAs from crude rice bran oil without any oryzanol loss in the extract fraction. Oryzanol content of the raffinate fraction was three times higher than that of the feed material. Phytosterol ester content of the

raffinate fraction was also increased during the process; however, their enrichment was not as high as that found for oryzanol (Dunford et al. 2003).

During the fractionation of rice bran oil, the effects of pressure (20–32 MPa) and temperature (45–80 °C) on the composition of the resultant fractions were examined by Dunford and King (2000) for isothermal operation of the column. The column was operated for 3 h in the semicontinuous mode, i.e. the feed was in a batch mode while CO_2 was added in continuous mode with CO_2 flow rate of 1.2 L/min. The amount of extract collected increased with pressure and temperature. The FFA content of the extracts was significantly higher than the raffinate samples. FFA content of the extracts, which were collected at 32 MPa, was lower than those obtained at lower pressures, because of the higher selectivity for triglycerides at high pressures. The extract with the highest FFA concentration (36.6 %) was obtained at 20.5 MPa and 80 °C. Triglyceride content of the extracts decreased significantly with temperature at lower pressures. Free sterol content of the extract samples was significantly higher than that of raffinate samples at all pressure and temperature conditions. However, sterol concentration of raffinate fractions (0.23–0.35 %) was still similar to that of the feed material (0.33 %). The amount of free sterol removed with the extract increased with increasing pressure and decreasing temperature. The oryzanol content of raffinate samples was significantly higher (3 to 5 fold) than that of extract fractions. The implication of this finding was reported to be quite important for the application of supercritical fractionation technology to rice bran oil de-acidification because rice bran oil which is refined using conventional process does not contain significant amount of oryzanol. The amount of oryzanol removed with the extract fraction was higher at low temperature and high pressure. Sterol fatty acid contents of the raffinate samples were significantly higher than those of the extract samples. Sterol esters removed by extract was high at high pressures but low at high temperatures. Low pressure—high temperature conditions were found to be favorable for minimizing triglyceride and phytosterol losses during the FFA removal from crude rice bran oil. As a result, rice bran oil with <1 % FFA, 95 % triglycerides and, 0.35 % free sterols and 1.8 % oryzanol content was obtained.

In the case of concentration of PUFAs from fish oils the processing scheme applied is column fractionation (Eltringham and Catchpole 2008; Catchpole et al. 2009b).

9.3.3 Extraction of Carotenoids

9.3.3.1 Solubility of Carotenoids in $SCCO_2$

Reported data on the solubility of carotenoids are very scarce and basically limited to the solubility of α- and β-carotene in $SCCO_2$ (Shi et al. 2007; Sovová et al. 2001).

9.3.3.2 Extraction Parameters

Available research on extraction of carotenoids from food industry by-products using $SCCO_2$ are summarized in Table 9.8.

Effect of Pressure. Pressure has a significant effect on the extraction yield of carotenoids by $SCCO_2$ extraction. Increasing pressure was reported to have significant positive effect on the α- and β-carotene yields from carrots (Sun and Temelli 2006), lycopene yield from tomato skins (Yi et al. 2009; Rozzi et al. 2002) but not on lutein yield from carrots (Sun and Temelli 2006). At 50 °C, the amount of β-carotene extracted from apricot pomace increased with increasing pressure from (20.2 to 30.3) MPa, then decreased with pressure increasing from (30.3 to 40.5) MPa (Şanal et al. 2005). Increasing the extraction pressure from (20 to 40) MPa with 10 MPa intervals resulted in a gradual increase in the yield of extraction and in the recovery of lycopene from tomato skin. However, further increase in pressure from (40 to 50) MPa did not improve the total amount of lycopene extracted (Topal et al. 2006). Similarly, Rozzi et al. (2002) reported a decrease in lycopene recovery from tomato skins and seeds with increase in pressure above 35 MPa. The maximum recovery achieved was 35 39%at 35 MPa and 86 °C .

Effect of Temperature. Temperature had a significant positive effect on the extraction yield of carotenoids by $SCCO_2$ extraction up to (80–90) °C. When temperature increased from (40 to 60) °C, the recovery of *trans*-lycopene from tomato seeds and skins increased but further rise in temperature to 80 °C led to a decrease in *trans*–lycopene recovery although the total lycopene (*cis*- + *trans*-) remained the same as that obtained at 60 °C (Nobre et al. 2009). Increasing temperature from (90 to 100) °C, at constant pressure of 40 MPa, provided almost the same amount of lycopene (1.18 mg/g) from tomato skin as there was degradation of lycopene at elevated temperatures (Topal et al. 2006). At constant pressure of 32 MPa, increasing temperature from (45 to 65) °C decreased the astaxanthin yield from blue crab shell waste from (57 to 17) % (Félix-Valenzuela et al. 2001).

Effect of Solvent Flow Rate. It was reported that lycopene yield from tomato skins and seed decreased with flow rate (Rozzi et al. 2002) but carotenoid yield from carrots was high at higher flow rate (Sun and Temelli 2006).

Effect of Cosolvents. Since the solubility of carotenoids is the lowest among the other minor lipid components, there is a need for use of cosolvents to increase their solubility. Among the solvents used are water, ethanol and vegetable oils. More soluble compounds (vegetable oils) are said to be acting as cosolvents (Fang et al. 2008).

At low cosolvent concentration (5 %), extraction yield of β-carotene from apricot pomace was higher when water was used rather than when ethanol was used as a cosolvent; however, at cosolvent concentration higher than 10 %, the extraction yield increased with increasing ethanol concentration but decreased with increasing water concentration. Maximum extraction yield was obtained at subcritical conditions (see Sect. 9.3.4). The optimum amount of β-carotene extracted (100.4 μg/g dry pomace) was predicted at 31.1 MPa, 69 °C and 27.4 % ethanol.

Table 9.8 Extraction of carotenoids from food industry by-products using SFE

By-product	Carotenoid	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Ref.
Apricot pomace	β-carotene	1	0.075–0.6	10.14	13–47	43–77	1 mL/min	90 min	2–28 vol.% ethanol 1–10 vol.% water	Şanal et al. (2005)
Apricot pomace	β-carotene	0.13–2	0.075–1.180	10–30	30–41	40–60	0.5–2 mL/min	90 min	–	Şanal et al. (2004)
Blue crab shell waste	Astaxanthin	13–25	<2	10.9	29.5–34.5	49–65	3.4–4.8 L/min	ni	10 % ethanol	Félix-Valenzuela et al. (2001)
Carrots	α-carotene, β-carotene, lutein	2	0.25–1.2	0.8–84.6	28–55	40–70	0.5–2 L/min	4 h	0–5 wt% canola oil	Sun and Temelli (2006)
Tomato skins	Lycopene	ni	1	7	20–40	40–100	1–2 mL/min	90 min	–	Yi et al. (2009)
Tomato skin	Lycopene	0.5	1	7	25–35	45–75	3.5 L/min	ni	0–20 % ethanol, water, olive oil or binary and ternary mixtures of them	Shi et al. (2009)
Tomato industrial waste (skins and seeds)	*trans*-lycopene	1.5	0.15–0.76	4.6–58.1	20–30	40–80	0.26–1.18 g/min	ni	–	Nobre et al. (2009)
Tomato skin and seeds	*trans*-lycopene	1.2	0.5–1	3	25–45	40–70	3.5 L/min	20 min S[a], 10 min D	5–15 % ethanol	Kassama et al. (2008)
Tomato pomace	Lycopene	100	ni	ni	37–53	47–53	0.7 L/min	0.17–1.84 h	16 % ethanol	Huang et al. (2008)

(continued)

Table 9.8 (continued)

By-product	Carotenoid	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Ref.
Tomato skin	Lycopene	ni	ni	ni	20–50	70–100	1.5–4.5 mL/min	330 min	–	Topal et al. (2006)
Tomato	Lycopene	ni	Cubed, 1	6, 60	34–45	45–66	8–20 kg/h	8 h	1–20 % hazelnut oil	Vasapollo et al. (2004)
Tomato skins and seeds	Lycopene, β-carotene	40–50	0.080–0.345	2	23–30	60–80	0.792–1.35 kg/h	ni	–	Sabio et al. (2003)
Tomato skins and seeds	Lycopene, β-carotene, tocopherols	3	ni	48	14–48	36–86	2.5–15 mL/min	ni	–	Rozzi et al. (2002)
Tomato skins	Lycopene	0.3	ni	ni	40	60–110	1.5 mL/min	80 min	550 μL acetone, methanol, ethanol, hexane, dichloromethane	Ollanketo et al. (2001)

[a]*S* static extraction, *D* dynamic extraction, ni not indicated

As demonstrated by Şanal et al. 2005, measured extraction yield at these conditions was 98 μg/g dry pomace; thus it increased by 32 % without adding cosolvent (74 μg/g dry pomace).

Using canola oil during the $SCCO_2$ extraction of carotenoids from carrot, improved the α-carotene and β-carotene yields more than twice and lutein yield more than four times compared to those obtained with $SCCO_2$ alone. The highest carotenoid yields were obtained at 55.1 MPa, 70 °C, 5 % canola oil concentration, (0.25–0.5) mm particle size, 0.8 % moisture content of the material and 2 L/min CO_2 flow rate. Total carotenoid yield was 1,904.3 μg/g feed. The α-carotene, β-carotene, lutein yields were 872.6, 905, 139.1 μg/g feed, respectively, at these conditions (Sun and Temelli 2006).

Among vegetable oils tested as cosolvents (almond, peanut, hazelnut and sunflower seed oils) for the extraction of lycopene from tomato, hazelnut oil gave the best results. The presence of cosolvent improved the yields of lycopene and had a beneficial role in the stability of the pigment with its lower acidity. At 45 MPa, 66 °C with 10 kg CO_2/h flow rate, addition of 10 % hazelnut oil in $SCCO_2$ increased the lycopene content in the extract from (10 to 32) % in 8 h of extraction. The maximum recovery of lycopene extracted at these conditions was 60 % (Vasapollo et al. 2004).

Shi et al. (2009) investigated the effects of adding ethanol, water and olive oil (single, binary and ternary mixtures), as cosolvents to $SCCO_2$, on the extraction of lycopene from tomato skins and demonstrated that the extraction efficiencies were improved. The highest yields were achieved at 35 MPa and 75 °C. For each of the three cosolvents applied yields increased with increased amounts (from 5 to 10 %) of the cosolvent. Thus, the highest lycopene yields were obtained with 15 % olive oil at 45 °C (50 μg/g) and with 10 % olive oil at 75 °C (80 μg/g). Among the binary and ternary mixtures of cosolvents, the mixture of ethanol (10 %) and olive oil (10 %) achieved the highest efficiency of 36.6 % at 45°C and of 56.8 % at 75 °C.

The optimized extraction parameters for carotenoid extraction from food industry by-products and carotenoid yield or recovery at these conditions are given in Table 9.9.

9.3.4 Extraction of Polyphenols

Polyphenols are conventionally recovered by solvent extraction. Ether, ethanol, methanol, ethyl acetate, acetone, water, ethanol/water and methanol/water mixtures are among the solvents used (Shi et al. 2005). Phenolic content of the extracts changes depending on the solvent used and on the type of the phenolics available in the extracted matrix. Besides the toxicity of the solvents applied, the phenolics might oxidize and isomerize as a result of heat and light during extraction. Furthermore, solvents need to be removed after extraction. Extraction rate (time) can be increased (decreased) by using microwave assisted extraction, ultrasound assisted extraction and high pressure liquid extraction. Although evaporation of

Table 9.9 Optimum extraction parameters of carotenoids from food industry by-products using SFE

By product	Carotenoid	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time	Cosolvent	Yield Recovery (%)	Ref.
Apricot pomace	β-carotene	1	0.075–0.6	10.14	31.1	69	1 mL/min	90 min	27.4 vol.% ethanol	100.4 μg/g	Şanal et al. (2005)
Apricot pomace	β-carotene	1	0.075–0.6	10.14	40.5	50	1 mL/min	90 min	–	88 μg/g	Şanal et al. (2004)
Carrots	α-carotene	2	0.25–0.5	0.8	55.1	70	2 mL/min	4 h	5 % canola oil	872.6	Sun and Temelli (2006)
Carrots	β-carotene	2	0.25–0.5	0.8	55.1	70	2 mL/min	4 h	5 % canola oil	905 μg/g	Sun and Temelli (2006)
Carrots	lutein	2	0.25–0.5	0.8	55.1	70	2 mL/min	4 h	5 % canola oil	139.1 μg/g	Sun and Temelli (2006)
Tomato skins	Lycopene	ni	1	7	40	100	1.5 mL/min	90 min	–	31.25 μg/g	Yi et al. (2009)
Tomato skin	Lycopene	0.5	1	7	35	75	3.5 ml/min	90 min	10 % olive oil	80 μg/g	Shi et al. (2009)
Tomato skin and seeds	*trans*-lycopene	1.2	0.5–1	3	45	62	3.5 mL/min	20 min S[a], 10 min D	14 % ethanol	23.9 μg/g 33 %	Kassama et al. (2008)
Tomato pomace (saponified)	Lycopene	100	ni	ni	40	57	0.7 L / min	1.8 h	16% ethanol	286.4 μg/g 93 %	Huang et al. (2008)
Tomato skin	Lycopene	ni	ni	ni	40	100	2.5 mL/min	330 min	–	1.18 mg/g	Topal et al. (2006)
Tomato	Lycopene	ni	1	6.6	45	66	20 kg/h	8 h	10 % hazelnut oil	60 %	Vasapollo et al. (2004)

[a]*S* static extraction, *D* dynamic extraction, ni not indicated

water is required after the extraction, a novel green process, namely subcritical water extraction (pressured hot water extraction) has been gaining importance in the recent years (Diaz-Reinoso et al. 2006; Junior et al. 2010; Pereira et al. 2010).

Extraction of polar substances can be achieved using $SCCO_2$ by adding cosolvents (5 wt% ethanol, methanol, water) to it in order to increase its solvating power or selectivity. Ethanol is the preferred cosolvent since it is GRAS and easily evaporated at room temperature. However, addition of a cosolvent to $SCCO_2$ increases the critical temperature of the binary mixture (Gurdial et al. 1993), which limits its amount in the supercritical region between (40–60)°C. Thus, a 5 wt% ethanol addition to CO_2 at any pressure above its critical point increases the critical pressure of the mixture to 42.5 °C. Hence, the extraction performed at this pressure and at 50 °C is called supercritical fluid (CO_2 + ethanol) extraction (SFE), while the extraction performed at the same pressure but at 40 °C is referred to as subcritical fluid (CO_2 + ethanol) extraction (SCFE). The critical temperatures of CO_2/ethanol mixtures containing (10, 14, 17, 20) wt% ethanol are (53.7, 62.8, 69.5 76.1) °C, respectively (Adil et al. 2007). Therefore, extractions performed between (40 and 60) °C with (15–20) wt% of ethanol addition to CO_2, are in the subcritical region of the mixtures.

Recovery of polyphenols from grape seeds, peels and pomace by using SFE or SCFE has been extensively studied (Palma and Taylor 1999a, 1999b; Murga et al. 2000; Pascual-Martí et al. 2001; Ashraf-Khorassanı and Taylor 2004; Louli et al. 2004; Cháfer et al. 2005; Pinelo et al. 2007; Yılmaz et al. 2011). Extraction of polyphenols from defatted or unfatted grape seeds, peels and pomace has been performed at pressures between (10 and 45) MPa at temperatures between (30–80) °C by addition of (5 to 20) % ethanol or methanol to CO_2. Extracted polyphenols have been limited by gallic acid, catechin, epicatechin and their derivatives (monomer flavanols), resveratrol (stilbene) and quercetin (flavonol). TPC and AA of the sour cherry pomace extracts obtained by SCFE with 20 wt% addition of ethanol to CO_2 at (20 to 60) MPa are lower than those of apple and peach pomaces (Adil et al. 2007, 2008). In another study, it was reported that defatted cherry pomace extracts, obtained at 25 MPa and at 50 °C with 10 % ethanol to CO_2, did not to contain any anthocyanins while the anthocyanin content of the extracts obtained with (20–40) % ethanol addition to CO_2 was half of the ethanolic extracts (Serra et al. 2010). These studies have shown that SCFE is not a good tool to recover polar polyphenols as anthocyanins from food industry by-products. The studies of polyphenols extraction from food industry by-products are summarized in Table 9.10.

9.3.4.1 Solubility of Polyphenols in $SCCO_2$

Phenolic acids and their esters are soluble in $SCCO_2$ without the addition of a cosolvent at (10 to 50) MPa and (40 to 60) °C (Choi et al. 1998; Murga et al. 2002, 2003, 2004; Diaz-Reinoso et al. 2006; Cháfer et al. 2007). Their solubilities are increased with the addition of (5 to 30) % ethanol in CO_2 (Diaz-Reinoso et al. 2006; Cháfer et al. 2007). The solubility of more polar phenolics as resveratrol (stilbene),

Table 9.10 Extraction of polyphenols from food industry by-products using SFE or SCFE

By-product	Polyphenol	Feed (g)	Particle size (mm)	Moisture content (%)	*P* (MPa)	*T* (°C)	Flow rate	Time (min)	Cosolvent	Supercritical Process	Ref.
Apple pomace	Total phenolics	1	0.638	14	20–60	40–60	2 g/min	10–40	14–20 wt% ethanol	SCFE	Adil et al. (2007)
Grape pomace	Gallic acid, catechin, epicatechin	100	ni	ni	8–35	35–50	0.2–1[a]	30–90	8 % ethanol	SFE/SCFE	Pinelo et al. (2007)
Grape seeds	Proanthocyanidins	30	0.328	ni	25–30	30–50	5 g/min	60	5–20 % ethanol	SFE/SCFE	Yılmaz et al. (2011)
Grape seeds[b]	Catechin, epicatechin	5	ni	ni	65.5	80	ni	60	30–40 % methanol	SCFE	Ashraf-Khorassanı and Taylor (2004)
Grape seeds[b]	Gallic acid, catechin, epicatechin	ni	ni	ni	20–30	40	ni	ni	2–15 vol.% ethanol, methanol	SFE/SCFE	Murga et al. (2000)
Grape seeds	Gallic acid, catechin, epicatechin	0.03	ni	ni	ni[c]	35–55	1 mL/min	20 S[d], ni D	10, 40 % ethanol, methanol	SCFE	Palma and Taylor (1999a)
Grape seeds[b]	Gallic acid, catechin, epicatechin	7.5	ni	ni	45	35	ni	15 S[d], ni D	20 vol.% methanol	SCFE	Palma and Taylor (1999b)
Grape skin	Catechin, epicatechin, resveratrol	1	ni	ni	25	60	2 mL/min	3 S[d], 15 D	20 vol.% ethanol	SCFE	Cháfer et al. (2005)
Peach pomace	Total phenolics	1	0.638	14	20–60	40–60	2 g/min	10–40	14–20 wt% ethanol	SCFE	Adil et al. (2007)
Sour cherry pomace	Total phenolics	1	0.638	14	20–60	40–60	2 g/min	10–40	14–20 wt% ethanol	SCFE	Adil et al. (2008)
Sweet cherry	Total phenolics, total anthocyanins	ni	ni	ni	25	50	ni	90	10–20 % ethanol	SCFE	Serra et al. (2010)

[a]Solid to solvent ratio
[b]Defatted by $SCCO_2$ extraction
[c]Pressures corresponding to CO_2density of (0.85–0.95) g/mL at (35–45) °C
[d]*S* static extraction, *D* dynamic extraction, ni not indicated

catechin, epicatechin (monomer flavanols), quercetin (flovonol) in $SCCO_2$ can be achieved at (8 to 12) MPa and at 40 °C with the addition of ethanol to CO_2 (Berna et al. 2001a, 2001b; Cháfer et al. 2002, 2004; Diaz-Reinoso et al. 2006).

Hydroxycinnamic acids are soluble in $SCCO_2$ at pressures between (8.5 and 50) MPa and at temperatures between (30 and 40) °C; among those, ferulic acid is the most soluble, caffeic acid is the least soluble one, while *p*-coumeric acid has solubility between those two (Murga et al. 2003). Quercetin, catechin and epicatechin are soluble in supercritical or subcritical (CO_2 + ethanol) (up to 30 %). Above 100 MPa and at 40 °C, epicatechin is more soluble than quercetin, and quercetin is more soluble than catechin (Cháfer et al. 2002, 2004; Berna et al. 2001b).

9.3.4.2 Pretreatment of By-Products

Before the extraction of polyphenols from industrial by-products, freeze dried and ground raw material is subjected to extraction with $SCCO_2$ (Serra et al. 2010; Ashraf-Khorassanı and Taylor 2004; Murga et al. 2000) in order to remove low polarity CO_2 soluble compounds, mainly oil in the grape or fruit seeds. This pretreatment using $SCCO_2$ is required in order to increase the phenolic yield.

9.3.4.3 Extraction Parameters

The most effective extraction parameters are pressure, temperature, co-solvent concentration and extraction time whereas the effect of fluid flow rate is minor.

Effect of Pressure. When $SCCO_2$ extraction is performed, pressure is the most significant parameter which affects the TPC (mg GAE/g extract) of the extracts (Gelmez et al. 2009). The TPC of wheat germ oil extracts decreased with increasing pressure. It is known that solubility of phenolics increases with increasing pressure resulting in an increase of phenolic yield. Although phenolic yield (mg GAE/g germ) increased with pressure, TPC of the extracts decreased because extraction yield increased at the same time. Pressure appears to have bigger effect on the solubility of wheat germ oil than on the solubility of phenolics, which is indicated by the decrease in phenolic concentrations (mg GAE/g extract) in the extracts with increasing pressure. Parallel to TPC of the extracts, pressure decreased AA of the extracts, as well.

During the SCFE of polyphenols from fruit pomaces, pressure had a significant effect on TPC and AE of the extracts up to about 50 MPa (Adil et al. 2007, 2008). This is mainly due to the increase in the density of CO_2, i.e. increase in the solvating power with increasing pressure. This is parallel to the solubility behavior of hydroxycinnamic acids in supercritical CO_2 at (8.5–50) MPa and (40–60) °C (Choi et al. 1998; Murga et al. 2003) and to the solubility behavior of catechin (Berna et al. 2001b) and epicatechin (Cháfer et al. 2002) in either supercritical or subcritical (CO_2 + ethanol) at (8–12) MPa and 40 °C. The solubility of epicatechin

in CO_2 containing 20 % ethanol increased almost four times when pressure was increased from (8 to 10) MPa at 40 °C (Cháfer et al. 2002). Similar results were obtained during the extraction of phenols from grape seeds (Murga et al. 2000). Pressure affected AE of the extracts in the same way.

Effect of Temperature. The solubilities of phenolic acids and their esters in $SCCO_2$ have been shown to increase with increasing temperature above the cross-over pressure which is below 15 MPa. At higher pressures (20–60 MPa) both phenolic yield and TPC of the wheat germ extracts increased with increasing temperature since temperature was shown not to have a significant effect on extraction yield (Gelmez et al. 2009).

Effect of Cosolvents. Cosolvent concentration was reported to be the most effective parameter during SCFE of polyphenols (Yılmaz et al. 2011). Solubilities of quercetin, catechin and epicatechin increase with increasing ethanol concentration from (5 to 30) % (Cháfer et al. 2002, 2004; Berna et al. 2001b). The solubility increase of polyphenols in CO_2, with the amount of ethanol added, depends on the interactions between the solute and the cosolvent. The solubility of quercetin in (CO_2 + ethanol) increases with ethanol concentration due to increased phenol-alcohol interactions (Cháfer et al. 2004). Although catechin presents a lower melting point than its isomer epicatechin, at 9 MPa and 40 °C, epicatechin has higher solubility in (CO_2 + ethanol) than catechin since its polar nature provides more hydrogen-bonding or dipole-dipole interactions with ethanol than catechin (Cháfer et al. 2002). The optimal concentration of ethanol was reported to be 7.5 % for the extraction of resveratrol from grape skins (Pascual-Martí et al. 2001), 10 % for the extraction of total phenolics from cherries (Serra et al. 2010), 15 % for the extraction of epicatechingallate, 20 % for the extraction of gallic acid, catechin, epicatechin, epigallocatechin, and epigallocatechingallate from grape seeds (Yılmaz et al. 2011), 20 % for the maximum extraction of total phenolics from apple, peach and sour cherry pomaces (Adil et al. 2007, 2008). Anthocyanins were not detected in $SCCO_2$ pretreated sour cherry extracts when 10 % ethanol-CO_2 mixture was used at 25 MPa and 50 °C. The concentration of anthocyanins increased to 0.08 mg cyanidin-3-glycoside equivalents (C3G) by increasing ethanol concentration in the mixture to 20 %. Enhanced solvent extraction with 40 % ethanol + CO_2 at the same conditions yielded 0.33 mg C3G/g, which corresponds to 33 % anthocyanin recovery compared to that of enhanced solvent extraction with pure ethanol (Serra et al. 2010).

Effect of Extraction Time. Oil recovery controls the phenolic concentration in the extracts when there are co-extractants like oil during extraction of phenolics. Time and pressure, up to about 45 min and 45 MPa, respectively, significantly increased extraction yield of wheat germ oil; for short extraction times (35–40 min), TPC of the wheat germ extracts decreased with pressure up to 45 MPa but for long extraction times (>40 min), TPC of the extracts increased with pressure above 45 MPa. However, the effect of time on AA of the wheat germ extracts was not parallel to its effect on TPC of the extracts. AA of the extracts decreased with extraction time. This might be due to the effect of different interactions involved between the parameters or more likely due to the phenolics that are extracted at

Table 9.11 Characteristics of the fruit pomace extracts obtained by SCFE at optimal extraction conditions (50–60 MPa, 50–55 °C, 2 g/min $SCCO_2$ flow rate, 20 % ethanol, 40 min extraction time) (Adil et al. 2007, 2008)

	Apple pomace	Peach pomace	Sour cherry pomace
SCFE			
TPC (mg GAE/g sample)	0.47	0.26	0.6
AE (mg $DPPH^{\bullet}$/g sample)	3.30	1.50	2.36
AE/TPC (mg $DPPH^{\bullet}$/mg GAE)	7.02	5.77	3.83
Ethanol extraction			
TPC (mg GAE/g sample)	1.71	0.81	2.92
AE (mg $DPPH^{\bullet}$/g sample)	9.30	6.21	24.80
AE/TPC (mg $DPPH^{\bullet}$/mg GAE)	5.44	7.67	8.49

these conditions having lower antioxidant activity compared to those extracted at lower pressures and extraction times (Gelmez et al. 2009). In the case of SCFE of cherries at 25 MPa and 50 °C with 20 % ethanol concentration prolonging extraction for 60 min after 90 min of extraction increased AA/TPC from 80 to 94 (Serra et al. 2010).

9.3.4.4 Characterization of Products

TPC and AA of wheat germ oil at optimum $SCCO_2$ extraction conditions are given in Table 9.2. TPC and AE of the extracts from apple, peach and sour cherry pomaces obtained by SCFE at optimized extraction conditions are reported in Table 9.11. It can be seen that TPC and AE of the extracts obtained from all pomaces are lower than those obtained by ethanol extraction (Adil et al. 2007, 2008). In apple, 95 % of flavan-3-ols is (−) epicatechin and 5 % is (+) catechin (Guyot et al. 1998). However, in peach the concentration of epicatechin is less than the concentration of catechin (Tomás-Barberán et al. 2001). Also, the concentrations of ferulic and *p*-coumeric acids in peach are less than the concentrations of those in apple (Leantowicz et al. 2002). Furthermore, anthocyanins are slightly soluble in subcritical (CO_2 + ethanol). Therefore, low TPC and AE of the extracts from peach pomace, compared to apple pomace extracts, can be explained by low concentrations of the more soluble hydroxycinnamic acids, epicatechin and the presence of anthocyanins in peach. AE/TPC of the extracts from apple pomace obtained by subcritical (CO_2 + ethanol) was higher than that of the extracts obtained by ethanol extraction indicating that less but more active polyphenols were selectively extracted by subcritical (CO_2 + ethanol) extraction compared to ethanol extraction. The low TPC of the extracts obtained from sour cherry pomace compared to ethanol extraction is due to its high anthocyanin concentration.

Yılmaz et al. (2011) reported that polyphenols were extracted from grape seeds at their maximum level when different parameters were used which was because of their different polarities. Gallic acid (32.9 ppm), epigallocatechin (218 ppm) and

epigallocatechingallate (47.17 ppm) were extracted at their maximum level at 30 MPa, 50 °C and 20%ethanol. Maximum amount of catechin (90.3 ppm) and epicatechin (43.1 ppm) were obtained at 30 MPa, 30 °C and 20% ethanol; 25 MPa, 30 °C and 15 % ethanol was needed to extract the highest amount of epicatechingallate (6.8 ppm).

9.4 Supercritical Antisolvent Extraction and Fractionation

Studies on recovery of phenolics by using either SFE or SCFE have revealed that organic solvents usage cannot be eliminated completely. In the light of this fact, conventional solvent extraction step is maintained for the recovery of phenolics and Supercritical Antisolvent Extraction (SAE) is used to completely remove the organic solvent and to precipitate the phenolics without a thermal degradation. Applications of SAE include the recovery of lecithin from soybean oil (Reverchon and de Marco 2006), recovery of polyphenols and anthocyanins from methanolic grape extracts (Floris et al. 2010). Supercritical Antisolvent Fractionation (SAF) has been used to fractionate natural matrixes which contain fat and water soluble biomaterials. Applications of SAF include concentration of PUFAs from fish and plant oils (Catchpole et al. 2009b), production of flavonoid concentrates from propolis (Cathpole et al. 2004), fractionation of various plant and animal extract solutions including by-products as grape seeds and skin (Catchpole et al. 2009a).

9.4.1 Processing Principles

SAE involves the continuous contact of liquid (bioactive compound and the organic solvent) and $SCCO_2$in a high pressure precipitator. The organic solvent dissolves in $SCCO_2$ and the bioactive compounds precipitates at the bottom of the precipitator. The solvent which dissolves in $SCCO_2$ is recovered in a low pressure separator (Floris et al. 2010).

SAF is developed by Catchpole et al. (2009a) for the fractionation of plant extract solutions using near critical fluids to give two or more fractions containing bioactives with widely different polarities. It is originally referred to as Supercritical Antisolvent Fractionation Technology (SAFT) by the authors. The extract solutions are obtained by a prior extraction of plant material using ethanol/water mixtures. One fraction is insoluble in the near critical fluid and is precipitated by antisolvent; the other fraction is soluble in the near critical fluid and cosolvent, and is recovered by downstream pressure reduction. With two stage pressure reduction, two fractions are obtained.

9.4.2 Processing Parameters

The parameters of SAE are pressure, temperature, molar ratio of CO_2 to organic solvent (i.e. $SCCO_2$ and liquid flow rates). The liquid is atomized through a nozzle or injected through a coaxial tube in the $SCCO_2$. Precipitation pressure and temperature should be above the critical pressure and temperature of the CO_2/liquid mixture. The precipitation takes place rapidly and efficiently above the critical point of the mixture. Liquid flow rate should be high enough to achieve the atomization and the jet formation. In this way the contact area between the liquid and $SCCO_2$, increases and therefore mass transfer increases. The CO_2 flow rate should be adjusted so that the molar concentration of the CO_2 is in the supercritical region of organic solvent/CO_2 mixture. Constituents like sugar, which might interfere with the mixture, should be removed before SAE (Floris et al. 2010).

Floris et al. (2010) extracted polyphenols from lyophilized grape residues using a tartaric buffer to avoid extract degradation due to polyphenol oxidase, hydrolysis and esterification, and to eliminate part of the interfering compounds, mainly sugars. The extract was selectively adsorbed on a C18 column and desorbed using methanol. The methanolic solution is processed by SAE. The pressure and temperature was selected as 11 MPa and 40 °C, just above the critical point of CO_2-methanol mixture which was at 8 MPa and 40 °C. CO_2 flow rate was 25 mL/min and methanolic solution flow rate was 0.7 mL/min.

The parameters controlling SAF are pressure, temperature, the solvent composition, concentration of the solids in the solution, flow rate ratio of solution to near critical fluid. In general, separation between highly polar bioactives, which are recovered in the insoluble fraction i.e. raffinate, and low to medium polarity fractions that are recovered in the extract fractions, is maximized at low soluble solids, water contents in the feed solution ≤ 30 wt%, and flow rate ratios of feed to $CO_2 \leq 30$ % when using CO_2 as an antisolvent fluid. The first separator is normally operated at conditions just above the critical point of ethanol/CO_2 mixtures typically around 9 MPa at 40 °C and 11 MPa at 60 °C. The second separator is operated at dense gas conditions at 5 MPa (Catchpole et al. 2009a).

9.4.3 Characterization of Products

Using SAE, extracts rich in anthocyanins were obtained from grape residues. At the optimized parameters of 11 MPa, 40 °C, CO_2 and methanolic solution flow rates 25 and 0.7 mL/min, respectively, the product is powder like and completely free of solvent. The total polyphenols extracted were 16,063 mg/kg where the amount of anthocyanins was 15,542 mg/kg of treated material. The most abundant polyphenols extracted were catechin (113.9 mg/kg), epicatechin (96 mg/kg), epicatechingallate (101.6 mg/kg) and, among the anthocyanins, malvin acetate (1,304 mg/kg) and malvin cumarate (9,256.9 mg/kg). Low and high polarity

fractions were obtained from grape seeds and skins by SAF process. Low polarity fractions contained grape oil from grape seeds and waxes from grape skins. High polarity fractions from both by-products were rich in anthocyanins (Catchpole et al. 2009a). PUFA concentrates containing more than 90 % ω-3 fatty acids were obtained from fish, seed, shark liver oils, and oil DODs (Catchpole et al. 2009b).

Conclusions

Supercritical fluid processing is an excellent tool for the recovery of bioactive compounds from food industry by-products. Specialty oils rich in polyunsaturated fatty acids and other bioactive compounds as tocopherols, phytosterols, carotenoids and polyphenols having high antioxidant activities are easily obtained by $SCCO_2$ extraction without the major need for addition of cosolvents. Depending on the purpose of $SCCO_2$ processing, oil or bioactive yields, or bioactive concentrations in the extracted oil can be optimized by changing the extraction parameters like pressure, temperature and extraction time. PUFAs or minor lipid components as tocopherols, phytosterols and squalene in the specialty oils (corn, rice, wheat barn oils, wheat germ oil, fish oil) or by-products (oil DOD) can be enriched by using $SCCO_2$ extraction, fractional extraction, fractional separation and column fractionation. For the extraction of carotenoids which are the least soluble minor lipid component in $SCCO_2$, SFE needs to be performed with the addition of cosolvents as ethanol. For the extraction of polar polyphenols, addition of large amounts of ethanol (10–30 %) is needed which makes the processing SCFE.

For the extraction of anthocyanins use of organic solvents cannot be avoided. This brings the alternative of using supercritical fluids as antisolvents. By using SAE and SAF polar polyphenols can be extracted and fractions with different polarities can be obtained. Future trends include particle formation with biomaterials or encapsulation of biomaterials with natural oil, starch or protein based materials. Supercritical antisolvent precipitation or co-precipitation can be used successfully for this purpose.

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Chapter 10
Supercritical Fluid Extraction of Compounds from Spices and Herbs

José A. Paixão Coelho and António M. Figueiredo Palavra

10.1 Introduction

Spices and herbs represent a substantial fragment of flavoring agents in food-beverage, cosmetic, perfume, and pharmaceutical industries in the world. Spices can be defined as "vegetable products used for flavoring, seasoning and imparting aroma in foods" (Douglas et al. 2005). Spices may be derived from many parts of the plant: bark, buds, flowers, fruits, leaves, rhizomes, roots, seeds, stigmas and styles or the entire plant tops. Herbs are leafy spices, and some, like dill and coriander, can provide both spice seeds and leafy herbs (Matthews and Jack 2011).

The antioxidant, antimicrobial, antimitotic and other medicinal properties of spices and herbs, offering extensive applications as natural compounds, are responding to the growing wave of consumer resistance and legislative limitations set for products containing chemical additives (Peter 2006). In fact, spices and herbs as sources of natural colors and flavors, bestow welcome opportunities in the international market.

Spices and herbal essential oils contain the volatile components presents in this plant matrices which are irresponsible for their characteristic aroma. On the other hand, the corresponding oleoresins embody its complete flavor profile. They contain the volatile, as well as non-volatile constituents of spices and can be obtained from them by extraction with a non-aqueous solvent.

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There are several techniques appropriate to isolate these compounds from plants. Methods like hydrodistillation, steam distillation and Soxhlet extraction have been employed for a long time. However, in recent years, new techniques have appeared, such as ultrasound-assisted extraction, microwave-assisted extraction, accelerated solvent extraction and supercritical fluid extraction (SFE).

SFE of plant material can be an important alternative to obtain natural compounds from spices and herbs. Most of the supercritical fluids are available in a relatively pure grade at a reasonable cost, as compared with the industrial grade liquid solvents. Their non-toxicity, non-flammability, as well as the ease of recovery and the selectivity of the extraction process, are the most important features of SFE (Mukhopadhyay 2001).

The knowledge about the positive effects of antioxidants against diseases involving oxygen reactive species is increasing. Therefore, at present, it is accepted that an antioxidant diet can be important for prevention of, for example, diabetes, cancer and Alzheimer's, Parkinson's and cardiovascular diseases. On the other hand, since antioxidants have the capability of reducing radical reactive species, they can also be applied in preventing food deterioration resulting from lipid oxidation. Furthermore, the processing and storage of food can lead to loss of endogenous antioxidants. So, their own protection is reduced against oxidation reaction (Laguerre et al. 2007).

Taking into account the advantages of antioxidants in food, at present a new type of food supplements, enriched with these compounds, are marketed (Herrero et al. 2006).

Due to incorrect use of antibiotics, experts from the World Health Organization (WHO) are deeply concerned with the emergence of multi-resistant strains of bacteria. Therefore, it is vital to mankind to discover new antibiotics that treat infections induced by this type of bacteria.

The search of natural antioxidants and antibacterial compounds has increased in recent years due to concerns about the use of toxic synthetic molecules. SFE is presently an important alternative to the traditional separation methods to obtain natural antioxidants and antibacterial compounds.

10.2 Classification of Spices and Herbs

Since October 2013 the European Spice Association (ESA) published a list of culinary herbs and spices mainly used and their regular botanical names in Europe (ESA 2013). ESA makes important considerations like the divergence of the botanical definitions from the traditional common use over the time. For instance, pepper is sometimes classified as fruit, while according to its botanical definition it is a seed; analogous is the situation with aniseed and other so-called seeds which are in fact fruits.

Although there are different types of classification of spices and herbs, one of the most widely-used is per indigenous climate zone: tropical zone, **TR**; temperate, **TE** and subtropical zone; **SU**. Table 10.1 shows examples of spices and herbs classification per climate zone, as well as the plant part used in the extraction.

10.3 Spice and Herbal Constituents

Secondary metabolites (extracts) are soluble fractions that can be removed from plant materials by dissolving the component(s) of interest in an aqueous, lipid, or alcoholic solvent, and in subcritical or supercritical fluid (mostly CO_2 or mixtures). Several of those compounds present in spices and herbs are antioxidants, and, after removal from the plant, can be added to food systems to prevent degradation, in particular, oxidation (Rojas and Brewer 2008; Sasse et al. 2009).

Antioxidant components of herbs and spices may be obtained as essential oils, resins or as extracts only. According to the European Pharmacopoeia (COE 2007), an essential oil is the extract obtained by distillation processes, like hydrodistillation (HD) and steam distillation (SD), with the exception of the *Citrus* sp. peel oil which is isolated by cold expression. When another technique is employed to extract the volatile fraction of an aromatic plant, an alternative name should be given. In the case of SFE the name volatile oil (VO) has been adopted in the literature to distinguish it that obtained by conventional methods.

The volatile fraction is a complex mixture of terpenoids, mainly monoterpenes (C10) and sesquiterpenes (C15), although diterpenes (C20) may also appear. However, a minor percentage of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters, lactones and even nitrogen and sulphur-containing compounds, coumarins and phenylpropanoids may also be present (Dorman and Deans 2000). Oleoresins, which include mainly the non-volatile fraction, which involve heavier molecular weight compounds, for instance, diterpenes (C20), triterpenes (C30), carotenoids (C40) phenylpropanoids and alkaloids, among others.

Table 10.2 shows examples of spices and herbs and the molecular structure of the respective bioactive compounds.

Phenolic compounds are the principal cause for the antioxidant activity of spices and herbs. The major antioxidant phenolic compounds from plants can be divided into four general groups: volatile oils (e.g. eugenol, carvacrol, thymol, and menthol); phenolic diterpenes (e.g. carnosol and carnosic acid), phenolic acids (e.g. gallic, protochatechuic, caffeic, and rosmarinic acids), and flavonoids (e.g. quercetin and catechin) (Shan et al. 2005; Brewer 2011).

10.4 Potential Bioactive Compounds from Spices and Herbs

There are numerous examples in the literature of the properties of bioactive compounds from spices and herbs. Black pepper (*Piper nigrum* L.) is one of the most widely used spices in the world, well known for its pungent constituent piperine. Piperine is the pungent compound of *Piper nigrum*, having been reported to possess immunomodulatory, anti-carcinogenic, antiasthmatic,

Table 10.1 Classification per climate zone of different herbs and spices, as well as the plant part used in the extraction, adapted from (ESA 2013)

Herbs and spices/common and botanical name	Climate zone	Herb or spice/part of plant used
Black pepper/*Piper nigrum*	TR	Spice/fruit
Basil/*Ocimum basilicum*	TE	Spice/leaves
Caraway/*Carum carvi*	TE	Spice/fruit
Cardamom/*Elettaria cardamomum*	TR	Spice/seed
Celeryseeds/*Apium graveolens*	TE	Spice/seed
Cinnamon/*Cinnamomum zeylanicum*	TR	Spice/inner bark
Cloves/*Syzygium aromaticum*	TR	Spice/flower buds
Coriander/*Coriandrum sativum*	TR	Spice/fruit
Cumin/*Cuminum cyminum*	TE	Spice/fruit
Fennel/*Foeniculum vulgare*	TE	Spice/fruit
Garlic/*Allium sativa*	TE	Spice/bulb
Ginger/*Zingiber officinale*	TR	Spice/rhizome
Nutmeg/*Myristica fragrans*	TR	Spice/seed
Peppermint/*Mentha piperita*	TE	Herb/leaf
Paprika/*Capsicum annuum or frutescens*	TE	Spice/fruit
Rosemary/*Rosmarinus officinalis*	TE	Herb/leaf
Wintersavory/*Satureja montana*	TE	Herb/leaf or whole
Saffron/*Crocussativus*	TE	Spice/parts of pistil
Thyme/*Thymus vulgaris*, *Thymus zygis*, *Thymus serpyllum*	TE	Herb/leaf or whole
Turmeric/*Curcuma longa*	TR	Spice/rhizome
Vanilla/*Vanilla planifolia*	TR	Spice/fruit

stimulatory, hepatoprotective, anti-inflammatory and antimicrobial activities (Meghwal and Goswami 2013).

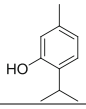
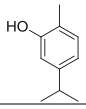
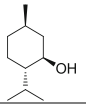
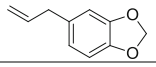
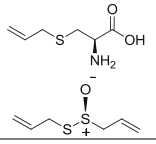
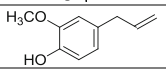
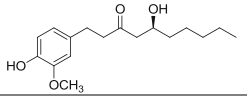
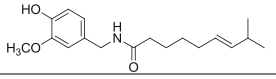
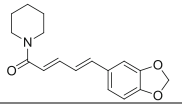
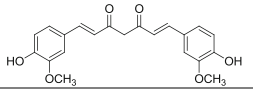
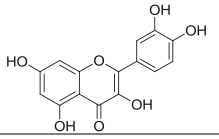
At present there is an increasing interest in food industry in the extraction of bioactive compounds from spices and herbs, due to their strong antioxidant and antibacterial activities.

1. **Antioxidant activity**
   During the 1940s the discovery of lipid oxidation inhibition by some synthetic antioxidants (phenolic compounds) has determined their use in food engineering. However, due to the controversy about their unsafe effects on human health, their consumption has decreased and is nowadays being replaced by natural antioxidants.

Considering the advantages of SFE against the traditional separation techniques (Bruno et al. 1993) several studies have been carried out to extract natural antioxidants from spices and herbs.

An interesting study on production of extracts with antioxidant properties was carried out involving the supercritical CO_2 extraction of volatile oil from winter savory—*Satureja montana* (Grosso et al. 2009b). The best compromise between

Table 10.2 Examples of spices and herbs presenting bioactive compounds and their respective structure

Spices and herbs/common and botanical name	Bioactive compound	Structures of the bioactive compound
Thyme, *Thymus vulgaris*	Thymol	
Savory winter, *Satureja montana*	Carvacrol	
Peppermint, *Mentha piperita*	Menthol	
Saffron, *Crocus sativus*	Safrole	
Garlic, *Allium sativa*	Allicin and S-allyl cysteine	
Clove, *Eugenia caryophyllus*	Eugenol	
Ginger, *Zingiber officinale*	Gingerol	
Red Pepper, *Capsicum annuum*	Capsaicin	
Black Pepper, *Piper nigrum*	Piperine	
Turmeric, *Curcuma longa*	Curcumin	
Onion, *Allium cepa*	Quercetin	

yield and composition when compared with essential oil was obtained at the temperature of 313 K, pressure of 9.0 MPa, flow rate of CO_2 of 1.1 kg/h and particle size of 0.6 mm. The extracts obtained presented the following main components: carvacrol (53 % in both cases), thymol (11 % in both cases), p-cymene (10.1 % for SFE and 12.8 % for HD), γ-terpinene(4.3 % SFE and

8.9 % HD) and β-bisabolene (2.5 % SFE and 2 % HD). However, one of the major differences between both oils is the presence of small percentage of waxes in the volatile oil, which doesn't seem to affect its quality and natural aroma. Another major difference is the content of thymoquinone, an oxygenated monoterpene with important antioxidant, anticancer and anti-inflammatory properties, which can be 15 fold higher in the volatile oil.

Taking into account the medicinal importance of *Satureja montana*, the nonvolatile fraction was also extracted (Silva et al. 2009). In fact, after SFE of all volatile oil, at the optimized experimental conditions, the plant residue was processed in the extractor at 25 MPa/313 K. In the first and second separators, operated, respectively, at 9 MPa/313 K and 2 MPa/293 K, two extracts E1 and E2 were obtained. To achieved the nonvolatile compounds, the plant residue (after the removal of volatile oil) was further processed by Soxhlet extraction with acetone (extract SE).

The health benefits from plants can be associated with the chemical profile in phenolic compounds (Celtković et al. 2007). HPLC screening of phenolic compounds in winter savory (*Satureja montana* L.) extracts, obtained by HPLC-DAD, is presented in Table 10.3. Extract SE contained the highest quantities of caffeic, gallic, gentisic and syringic acids and (–) epicatechin. On the other hand, in the extract E2 (+) catechin and protocatechuic, chlorogenic, vanillic, and ferulic acids were predominant. Moreover, in the extract E1 only the coumaric acid content is higher than the other SFE extracts.

Other antioxidant compounds have seen their importance in food industries being recognized. In 2009 the Environmental Protection Agency (EPA) reviewed the literature and published research on the toxicology and environmental impact of thymol and concluded that "Thymol has minimal potential toxicity and poses minimal risk". It has determined that thymol is a normal constituent of a human diet. At present thymol is listed as food additive by the FDA (21 CFR 172.515) and

Table 10.3 Relative percentage of phenolic compounds detected in three extracts from the nonvolatile fraction of *Satureja montana* determined by HPLC-DAD at 280 nm (adapted from Silva et al. 2009, Table 2)

Phenolic compound	Extract, E1 (%)	Extract, E2 (%)	Extract, SE (%)
Protocatecheuic acid	0.18	0.38	0.03
Chlorogenic acid	0.60	0.75	0.29
Gallic acid	1.35	0.14	1.93
Gentisic acid	0.10	0.03	0.37
Vanilic acid	0.21	1.03	0.38
Caffeic acid	0.46	0.21	0.92
(+)-Catechin	0.25	0.67	0.25
(–)-Epicatechin	0.20	0.75	0.80
Syringic acid	0.32	0.19	0.91
Ferrulic acid	0.40	0.42	0.29
Coumaric acid	0.52	0.22	0.39

E1 supercritical fluids extract from first separator, *E2* supercritical fluids extract from second separator, *SE* Soxhlet extract

thyme oil is recognized as generally accepted as safe (GRAS) essential oil by the FDA (21 CFR 182.20) as well (Federal Register 2009).

Table 10.4 presents a comparison of SFE with other methods of extraction used in the production of extracts with antioxidants activity from important spices and herbs.

2. **Antimicrobial activity**

   Studies on spices and herbs have been carried out to discover new molecules or a group of molecules that can be used as antibiotics, without the toxicity of the synthetic chemical compounds presently applied in the treatment of infections caused by multi-resistant strain bacteria.

Table 10.4 Supercritical extracts from spices and herbs with antioxidant activity and comparison with alternative extraction methods

Spices/herbs	Extraction method	Reference
Black cumin (*Nigella sativa*)	CO_2-SFE vs. SE (hexane or methanol)	Rao et al. (2007)
Black pepper (*Pipernigrun*)	CO_2-SFE vs. HD	Topal et al. (2008)
Boldo (*Peumusboldus*)	SWE, CO_2-SFE vs. CO_2-SFE + co-solvent (ethanol) vs. SE (methanol)	Del Valle et al. (2005)
Cardamom (*Elettaria cardamomum*)	Subcritical, CO_2-SFE, CO_2-SFE + co-solvent (ethanol) and SPE vs. M (1,2-dichloroethane–acetone–methanol, 2:1:1)	Hamdan et al. (2008)
Clove (*Eugenia caryophyllata*)	CO_2-SFE	Ivanovic et al. (2013)
Coriander (*Coriandrum sativum*)	CO_2-SFE vs. SE (ethanol)	Yepez et al. (2002)
Cumin (*Cuminum cyminum*)	CO_2-SFE vs. HD	Topal et al. (2008)
Fennel (*Foeniculum vulgare*)	CO_2-SFE vs. HD	Topal et al. (2008)
Ginger (*Zingiber officinale*)	CO_2-SFE vs.CO_2-SFE + co-solvent (ethanol and/or isopropyl alcohol)	Leal et al. (2003)
Laurel (*Laurus nobilis*)	CO_2-SFE + co-solvent (ethanol)	Santoyo et al. (2006a)
Marjoram (*Marjorana hortensis*)	CO_2-SFE vs. HD + M (acetone or water), M (acetone) + M (methanol/water mixture)	Dapkevicius et al. (1998)
	CO_2-SFE vs. HD	El-Ghorab et al. (2004)
Oregano (*Origanum vulgare*)	CO_2-SFE vs. HD + M (acetone or water), M (acetone) + M (methanol/water mixture)	Dapkevicius et al. (1998)
	CO_2-SFE vs. CO_2-SFE + co-solvent (ethanol)	Cavero et al. (2006)
	SWE	Rodríguez-Meizoso et al. (2006)
	CO_2-SFE	Ivanovic et al. (2013)

(continued)

Table 10.4 (continued)

Spices/herbs	Extraction method	Reference
Rosemary (*Rosmarinusofficinalis*)	CO_2-SFE vs. UAE (acetone, methanol, hexane, or dichloromethane)	Tena et al. 1997
	CO_2-SFE vs. CO_2-SFE + co-solvent (ethanol and/or isopropyl alcohol)	Leal et al. 2003
	CO_2-SFE vs. CO_2-SFE + co-solvent (ethanol)	Ibáñez et al. (2000), Cavero et al. (2005)
	CO_2-SFE vs. HD, SE (hexane or ethanol)	Carvalho et al. (2005)
	CO_2-SFE vs. HD	Topal et al. (2008)
	CO_2-SFE	Chang et al. (2008)
Sage (*Salvia officinalis*)	SWE vs.UAE (methanol) vs. HD and M(70 % ethanol)	Ollanketo et al. 2002
	UAE (petroleum ether and 70 % (v/v)aqueous ethanol solution)	Kaufmann and Christen 2002
	CO_2-SFE vs. HD + M (acetone or water), M (acetone) + M (methanol/water mixture)	Dapkevicius et al. 1998
Thyme (*Thymus vulgaris*)	CO_2-SFE vs. HD + M (acetone or water), M (acetone) + M (methanol/water mixture)	Dapkevicius et al. (1998)
	CO_2-SFE vs. HD, SE (hexane or ethyl alcohol)	Simandi et al. (2001)
	UAE (dichloromethane or ethanol) vs. M with ethanol and HD	Chizzola et al. (2008)
	CO_2-SFE vs. HD	Topal et al. (2008)
	CO_2-SFE vs. HD	Grosso et al. 2010
Turmeric (*Curcuma longa*)	CO_2-SFE vs. CO_2-SFE + co-solvent (ethanol and/or isopropyl alcohol)	Leal et al. (2003)
Summer savory (*Satureja hortensis*)	CO_2-SFE vs. HD	Esquível et al. (1999)
Winter Savory (*Satureja montana*)	CO_2-SFE vs. HD, SE (acetone or pentane)	Silva et al. (2009)

CO_2-SFE supercritical fluid extraction with CO_2, *ASE* accelerated solvent extraction, *UAE* ultrasound-assisted extraction, *SWE* sub-critical water extraction, *SPE* sub-critical propane extraction and comparison with conventional techniques, *HD* hydrodistillation, *SE* Soxhlet extraction, *M* maceration

An experimental study of the antibacterial activity of the volatile oil, A, and essential oil, B, from *Satureja montana* suggests a great potential for the growth control and inactivation of *Bacillus subtilis* and *Bacillus cereus*as has shown in Table 10.5 (Silva et al. 2009).

The antibacterial activity of the volatile oil fraction (A and B extracts) was higher than that shown by the nonvolatile fraction, with the exception of SFE extract E2. The volatile oil and non-volatile extracts A and E2, respectively, both obtained by SFE, have antibacterial activity superior to *Bacillus cereus* and *Bacilluss ubtilis*.

Table 10.5 Antibacterial activity of *Satureja montana* extracts: SFE versus conventional extraction (adapted from Silva et al. 2009, Table 4)

Saturejamontana extracts					
	Volatile fraction		Nonvolatile fraction		
	CO_2-SFE (A)	HD (B)	CO_2-SFE (E1)	CO_2-SFE (E2)	SE (D)
Bacteria					
Bacillus cereus	20	10	–	19	–
Bacillus subtilis	47	12	11	34	–
Enterococcus faecalis	11	10	–	–	–
Escherichia coli	11	11	–	–	8
Listeria monocytogenes	11	12	–	11	–
Pseudomonas aeruginosa	–	–	–	–	–
Salmonella enteritidis	11	–	–	–	–
Staphylococcus aureus	10	11	–	9	–

Results were determined using the paper disk diffusion method and are expressed by the diameter of the inhibition zones (mm); (–) inhibition diameter <6.4 mm. *CO_2-SFE* supercritical fluid extraction with CO_2, *HD* hydrodistillation, *SE* Soxhlet extraction

Moreover, beyond the studies on antioxidant and antibacterial activities of supercritical extracts from *Satureja montana*, studies on anticholinesterase activity have also been carried out. This study has shown particularly interesting results concerning butyrylcholinesterase inhibition. In fact, the supercritical extract E2 has shown to be the most promising inhibitor of this enzyme. In contrast, the extract SE at the concentrations tested didn't affect the activity of this enzyme (Silva et al. 2009).

Other studies have been carried out to screen the possible antimicrobial activity of supercritical fluid extracts towards different microbial species, including gram positive bacteria, gram negative bacteria, yeast (e.g. *Candida albicans*) and fungus (e.g. *Aspergillus niger*). These results are reported in Table 10.6. The solvent being utilized was CO_2 or CO_2 + co-solvent (ethanol), and compared principally with hydrodistillation and Soxhlet extraction.

In 2013, extracts from clove buds, *Eugenia caryophyllus*, obtained by supercritical carbon dioxide extraction were screened for antioxidant and antibacterial activities (Ivanovic et al. 2013). Additionally, antioxidant and antibacterial activities of these extracts obtained by SFE of the clove bud–oregano leaf mixtures were carried out. The extracts of clove, within the presence of oregano extract, show an antioxidant activity comparable to synthetic antioxidants. The antibacterial activity of clove extract has presented a moderate effect in *Staphylococcus* and *Enterococcus* bacterial strains. However, an improvement of antibacterial activity of clove extract against all tested strains, was observed when 50 % of the oregano extract was present, resulting in a synergistic antibacterial activity against Methicillin-resistant *Staphylococcus haemolyticus* strain.

Table 10.6 Supercritical fluid extracts from spices and herbs with antimicrobial activity

Species/herbs	Antibacterial activity/bacterial species	Reference
Cassia Cinnamon (*Ramulus Cinnamomi*)	*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	Liang et al. (2008)
Garlic (*Allium sativa*)	*Bacilus subtilis, Candida utilis, Escherichia coli, Pseudomonas aurantiaca*	Zalepugin et al. (2010)
Kaffir lime (*Citrus hystrix*)	*Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Malassezia furfur*, *Propionibacterum acnes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*	Pyo and Oo (2007)
Laurel(*Laurus nobilis*)	*Aspergillus niger, Bacillus Subtilis, Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*	Santoyo et al. (2006a)
Marjoram (*Origanum majorana*)	*Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescens*	Vagi et al. (2005)
Oregano (*Origanum vulgare*)	*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*	Santoyo et al. (2006b)
	Escherichia coli, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*	Karakaya et al. (2011)
Rosemary (*Rosmarinus officinalis*)	*Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	Santoyo et al. (2005)
	Bacillus cereus, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	Genena et al. (2008)
Winter Savory (*Satureja montana*)	*Alternaria alternata*, *Bacillus cereus*, *Bacillus subtilis*, *Biscogniauxia mediterrânea*, *Botrytis* spp., *Candida albicans*, *Colletotrichum coffeanum*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Pyricularia oryzae*, *Pseudomonas aeruginosa*, *Rhizopus* spp., *Salmonella enteritidis*, *Staphylococcus aureus*, *Stachybotrys chartarum*	Silva et al. (2009)

10.5 Process Parameters

The capabilities of SFE, namely SFE with CO_2 ($SCCO_2$), has been compared with conventional extraction methods in terms of selectivity, and possibility of manipulating the composition of the extract. However, the discussion of the application of $SCCO_2$ extraction in the isolation of volatile oils and oleoresins from selected spices and herbs can only be performed with the correct selection and optimization of several parameters:

(1) Matrix parameters, like particle size, shape, surface area, porosity, moisture and the nature of the matrix.
(2) Operating parameters, such as, pressure, temperature, flow rate and the presence of the so called modifiers.

Moreover, the viability of the extraction and recovery system used should be seriously assessed in order to obtain the final desired product (one or more collection vessels). An interesting critical analysis of the works published on SFE of

volatile oils and other related products, giving a comprehensive study of the influence of the parameters, equipment and mathematical models of fragrance compounds can be found in Reverchon 1997.

In a study, assessing the process parameters required for economic analysis, Meireles 2003 examined process design data to perform supercritical extraction from solid matrices using carbon dioxide, with and without addition of co-solvent. The author concludes that, since many data are available, some systematization is missing about the nature of solid substratum and parameters.

Several authors (Herrero et al. 2006; Reverchon and De Marco 2006; Suhaj 2006; Pourmortazavi and Hajimirsadeghi 2007; Sovová and Stateva 2011; Martin et al. 2012) have reviewed the importance of the applications of SFE, used to isolate natural products from different raw materials, with special attention to the extraction of the most common and most-used spice and herbal antioxidants and methods of their preparation. Moreover, several of those works describe antioxidant/antiradical properties of the extracts, due to their important role in food preservation and health promotion. The principles and instrumentation, mathematical modeling and the elements of thermodynamic modeling background, designed to predict and model robustly and efficiently the phase equilibria of the systems solute plus supercritical fluid were also considered.

Recently, a review concerning SFE process parameters, such as pressure, temperature, solvent flow rate, size of grinding materials, and ratio of the co-solvent were presented for 19 selected spice plant materials. The range of operating conditions spans pressures from 7.5 to 68 MPa, temperatures from 293 to 363 K, solvent flow rate from 0.003 to 30.0 kg/h, and diameter of grinding material from 0.17 to 3.90 mm, representing the work developed in the last years (Sovilj et al. 2011).

Additionally, a comparison of the extraction yield and composition of the volatile oil of seven plants and herbs from *Lamiaceae* family, as well as the biological activity of SFE extracts by describing their insecticidal, acaricidal, antimycotic, antimicrobial, cytotoxic and antioxidant properties have been discussed (Fornari et al. 2012; Capuzzo et al. 2013). Moreover, these authors discussed the process modeling, mass-transfer mechanisms, kinetic and thermodynamic parameters, providing an overview of SFE potential in this field.

10.5.1 SFE Equipment

Solid processing is always done in a discontinuous (batch), or semi-continuous process (semi-batch), while liquid processing is usually carried out under counter-flow conditions in a continuous mode. In the semi-batch processing, which is widely employed, raw materials are introduced into the extractor to obtain a fixed bed of particles, the supercritical solvent being fed continuously by a high-pressure pump at a fixed flow rate.

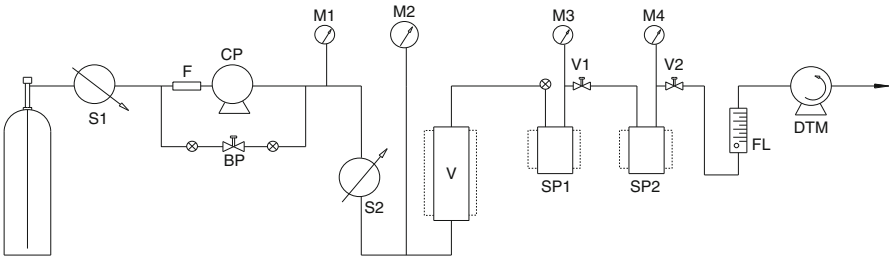


Fig. 10.1 SFE semi-pilot plant. S1, Ice cooler; F, filter; CP, circulating pump; BP, backpressure regulator; M1–M4, manometers; S2, heat exchanger; V, extraction vessel; SP1 and SP2, separators; V1 and V2, valves; FL, flow meter; DTM, dry test meter

An example of a SFE apparatus is shown in Fig. 10.1 and has been described in detail elsewhere (Reis-Vasco et al. 1999). It is composed of an extraction vessel, V, (1 L) and two separators (0.27 L), which operate in series, SP1 and SP2. The CO_2 is compressed by a circulation pump, CP and the pressure inside the extractor is controlled by a back-pressure regulator and measured with a Bourdon-type manometer, M. A pre-set temperature in the extractor is reached with the aid of a water jacket. The total volume of CO_2 is determined with a dry testmeter, DTM.

Usually one or more separation stages are used to precipitate the solute from the supercritical solution in flash vessels (cyclonic and gravimetric separators) usually changing drastically the solvent power of CO_2 by depressurization or temperature change or both (Reverchon and De Marco 2006; Pronyk and Mazza 2009). In this case, it is possible to fractionate the extract in two or more fractions of different composition by setting adequate temperatures and pressures in the separators. With this strategy it is feasible to obtain volatile oil, oleoresin or other extracts in the different separation vessels according to the particular spices and herbs as reported (Coelho et al. 2012). These studies confirmed that SFE can produce superior quality products characterized by the absence of undesirable compounds and by a better reproduction of the original flavor or fragrance.

Moreover, recycling the solvent in pilot plants or larger systems are other important procedures in this case. The last separation stage has to be maintained at a suitable pressure and temperature conditions to condense the solvent and thus to facilitate the subsequent recompression.

10.5.2 Matrix Parameters

Matrix parameters such as particle size, shape, surface area and porosity, localization of extractable solutes, moisture content, drying effect and environmental agents are factors that depend on the nature of the matrix, or on pre-treatment of the plant material and will affect the SFE results.

10.5.2.1 Localization of the Extractable Solutes and Particle Size of the Matrix

Essential oils are synthesized and stored inside specialized structures. Two localizations are normally considered: at the surface of the plant organ (external secretory structure), or more deeply, closed to the vascular tissue (internal secretory structure).

These types of secretory structures, trichomes (external secretory structure) can be found in *Mentha pulegium* (Reis-Vasco et al. 1999), *Satureja fruticosa* (Coelho et al. 2007), *Satureja montana*, *Santolina chamaecyparissus* and *Thymus vulgaris* (Grosso 2010), while *Foeniculum vulgare* (Coelho et al. 2003) and *Coriander sativum* possess secretory ducts (internal secretory structure) (Grosso 2010; Mhemdi et al. 2011). Figures 10.2 and 10.3 are representative of these two kinds of secretory structures and show the decrease of particle size after the milling process.

These considerations, and not only the nature of the supercritical fluid or choice of extraction parameters, should be taken into account to the SFE. The interactions between solutes and active sites of the matrix can demand serious extraction conditions and the accessibility of the solvent to the solute presents a prominent importance.

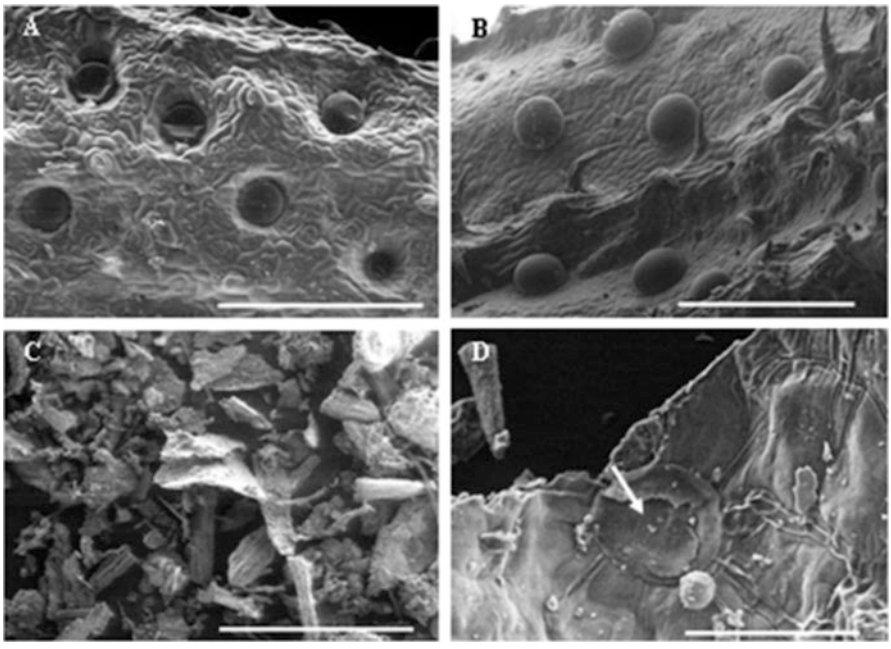


Fig. 10.2 SEM of *S. montana* aerial parts. (**A**) General view of the adaxial face of the leaf showing peltate trichomes (bar = 300 μm; ×150); (**B**) general view of the abaxial face of the sepal with peltate trichomes (bar = 300 μm; ×150); (**C**) grounded leaf portions (bar = 1 mm; ×50); (**D**) damaged peltate trichome in a rounded leaf portion (*arrow*) (bar = 50 μm; ×900). (From Grosso et al. 2009a, Fig. 3, published with kind permission of Journal of Separation Science 2013. All Rights Reserved)

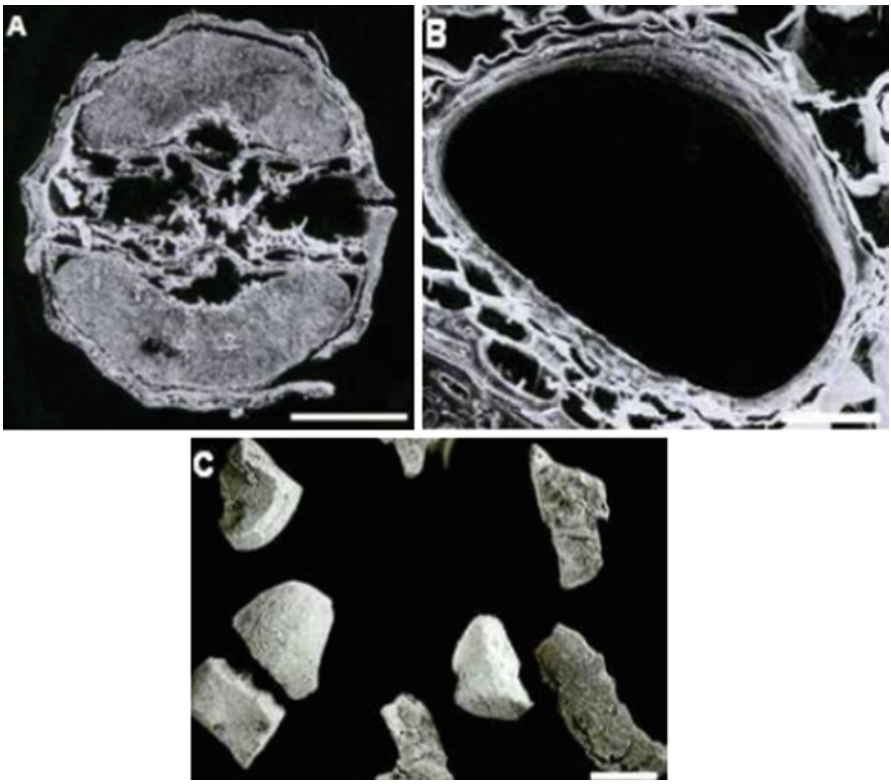


Fig. 10.3 Scanning electron microscopy images of coriander seeds. (**A**) Transversal section of coriander seed (bar = 1 mm); (**B**) ventral duct (bar = 50 μm); (**C**) particles obtained after seed grinding in 1 mm sieve (bar = 1 mm). (From Grosso et al. 2008, Fig. 3, published with kind permission of Food Chemistry 2013. All Rights Reserved)

The crushing of the matrix assumes some loss of the more volatile compounds in the plants. One method associated with SFE was suggested to disrupt trichomes cuticle, which eliminates the losses of volatile oils observed when mechanical treatments are used (Gaspar et al. 2001). The method consists of the application of compressed CO_2, followed by a rapid decompression process prior to the extraction. The disruption occurs, because during the initial pre-expansion period, in which the sample is exposed to high pressure of CO_2, the solvent slowly penetrates the glandular trichomes through the cuticle and then dissolves in the volatile oil, becoming saturated. When decompression of the plant particles takes place, the pressure within the trichomes also decreases and CO_2 starts to be desorbed. However, due to the resistance found in the cuticle, CO_2 plus volatile oil are entrapped inside the subcuticular space and an excess of pressure inside it is generated. Thus, a pressure gradient across the gland is formed, due to the inability of the glands to discharge the CO_2 and the disruptions occur. The efficiency of the process is improved when higher pressure gradients are applied.

If the SFE process is controlled by an internal mass transfer resistance, the diffusion of the supercritical solvent inside the plant particles is an important factor and it is essential to reduce the diffusion length by decreasing the plant particle size to facilitate such diffusion. The crushing is used, therefore, with two main objectives: to disrupt the cuticle of the trichomes and to reduce the CO_2 path length in particles with secretory ducts (since they are located internally).

However, if particles are too small (below 0.3 mm), preferential pathways to CO_2 may be formed inside the plant bed, due to compaction, and, as a result, part of the solvent flows through channels, leading to a loss of efficiency. Hence, the yield of the extraction is decreased (Reverchon and De Marco 2006; Grosso 2010). This channeling effect can be avoided if (Germain et al. 2005; Del Valle et al. 2006):

- Upflow mode of the supercritical fluid is chosen, as it prevents agglomeration and deformation of particles.
- The extractor used has a small diameter compared with its length, since this condition promotes a uniform flow.
- The mixing of plant particles with a dispersant, like glass beads, is performed.
- Stainless steel frits at the end of the extractor are used to cause uniform flow.
- And if the extraction is carried out with a low CO_2 flow rate to promote the diffusion of the solvent inside the pores within the sample instead of passing around the solid matrix.

A study about particle sizes effect on SFE process to obtain volatile oil from coriander seeds was carried out (Grosso 2010). A relationship was found to be established between the decrease of the particle size of coriander seeds and the increase of the volatile oil obtained. Internally, the oil is secreted in ducts located in the pericarp of the fruit (Fig. 10.3b) and these ducts are damaged during grinding, leading to the release of the oil, which produces an external film around the endosperm particles (Fig. 10.3c). So, the highest yield was obtained for the smallest particle size, indicating that more ducts were damaged and, therefore, the oil was more accessible to supercritical carbon dioxide.

For the system parsley seed oil–CO_2 under different conditions the effect of particle size on the extraction rate was also studied (Louli et al. 2004a). The extraction rate increases by decreasing the size of the seeds. The authors attribute this behavior to the higher amount of oil released as the seed cells are destroyed by milling. Moreover, after milling, the diffusion paths in the solid matrix become shorter resulting in a smaller intra-particle resistance to solute diffusion. This kind of relationships has been established several times in the literature.

10.5.2.2 Drying Effect

The drying of spices or herbs is one of the main preservation processes, which can be carried out conventionally by air-drying (with or without forced heat), or by freeze-drying. It is obvious that the drying process may have an influence on the content of aroma compounds.

Rosemary leaves obtained by different methods of drying have been submitted to SFE and antioxidant activity and volatile oil yield were evaluated, as well as the composition (Ibáñez et al. 1999). Two main factors were considered: the drying process, which influences the volatile oil composition and therefore the extract quality, and the effect of the drying process on the plant cells. The authors proposed a two-step SFE of rosemary leaves at selected conditions of pressure and temperature to divide the oleoresin into two fractions with different antioxidant activities and composition.

In conclusion, this study shows a great influence of both type of rosemary and drying treatment on the final results. It seems clear that drying at ambient temperature, in a ventilated place, is the method that provides better results. A loss of an antioxidant activity (higher with greater thermal treatment used) was reported, as well as a modification in volatile oil composition with reference to fresh rosemary.

Considering the challenge for the food industry to obtain quality products at a minimum cost and maximum yield, through optimal operating conditions, studies were carried out with *Thymus vulgaris*, considering the effect of drying process on antioxidant capacity of the thyme (Rodrigues et al. 2003). These researchers conclude that is necessary to shorten the heating time (drying time) to maximize the antioxidant capacity; which depends on air velocity and temperature of the drying process.

SFE of compounds from *Capsicum* peppers at temperatures of 313, 323 and 333 K and pressures of 15, 25 and 35 MPa was carried out to determine the best conditions of temperature in order to achieve the highest global yield and capsaicinoid content of the extracts (Aguiar et al. 2013). The influence of drying process (either freeze or oven drying) on total yield, capsaicinoids content and total phenolics was also analyzed. The freeze drying process resulted in extracts with the highest concentration of capsaicinoids (61 mg/g extract); but in contrast, the phenolics were less susceptible to the different drying processes with a mean concentration of 35 mg GAE/g extract.

10.5.2.3 Moisture Content

Moisture content of the solid material influences not only the extraction quality and yield, but also the fluid dynamics of the solvent, representing other important matrix parameter. To reduce water content and consequently drying the raw material it is usual to obtain a value of around 4–14 %. Water can compete with the solutes to be extracted and also acts as co-solvent by interacting with the supercritical solvent by changing the overall polarity of the fluid. Nevertheless, extracted water can increase the formation of ice blockages (Capuzzo et al. 2013). In fact, the solubility of water in CO_2 (0.3 %) can cause restrictor plugging upon the fluid depressurization, including the pressure of water in the collection system (Pourmortazavi and Hajimirsadeghi 2007).

Moreover, if excess water remains in the extractor, a highly water soluble solute will prefer to partition into the aqueous phase decreasing the capacity of SFE to

extract it. For solutes that are insoluble in water, it can precipitate onto matrix surfaces. Moreover, even though the solute may be very soluble in the extraction fluid, the excess water in the sample acts as a barrier to its transfer to the fluid (Lehotay 1997).

10.5.2.4 Environmental Factors

Environmental agents can affect the composition and essential oil content of matrix. For instance, a study carried out on the volatile oils from rosemary and sage obtained from several suppliers of the British market demonstrated that their antioxidant properties depend on its geographical location and type of processing (Svodoba and Deans 1992).On the other hand, four oregano plant species, collected during flowering season from 33 sites located in the eastern part of a Greek island, were evaluated on the basis of the essential oil constituents, and the results discussed in terms of topography and climatic variation, demonstrating the existence of two main groups, divided in four subgroups of taxo, were evidenced (Economou et al. 2011).

The evaluation of the influence of light intensity (plants exposed to direct sun and in controlled lighting conditions), and the age of leaves (6–24 months) on the characteristics of $SCCO_2$ extracts of mate tea leaves, obtained at 17.5 MPa and 303 K, has been carried out. Samples of mate were collected in an experiment conducted under agronomic control at Industria e Comercio de Erva-Mate Barão, Brazil. Quantitative analysis of caffeine, theobromine, phytol, vitamin E, squalene, and stigmasterol was performed, and the results showed that field variables exert a strong influence on the yield and the chemical distribution of the extracts (Esmelindro et al. 2004).

10.5.3 Operating Parameters

The influence of several operating parameters such as pressure, temperature, particle size and CO_2 flow rate on the SFE extraction yield and composition of the volatile oil and oleoresins should be considered and compared with those obtained by conventional methods (essential oils or oleoresins).

10.5.3.1 Pressure and Temperature

At constant pressure, the density of CO_2 decreases as temperature increases. Simultaneously, the temperature also affects the volatility of the solute. Hence, the effect of temperature elevation is difficult to predict due to its dependence on the nature of the sample. Moreover, at constant temperature the density of CO_2 increases when pressure rises. However, high pressure is not always recommended

for complex matrices, due to the higher solubility of solutes when the pressure is elevated, resulting in complex extracts and therefore difficult analysis. On the other hand, it should be considered that the presence of co-extracted solutes can dramatically change the solubility level of the solute with interest (Pourmortazavi and Hajimirsadeghi 2007).

A compromise between solvent power and solvent selectivity should be taken into account during SFE of compounds, since the raising of density leads to a reduction of selectivity. Therefore, the co-extraction of other undesirable compounds may occur. This is of particular importance for the volatile fraction. So, fractional separation of the extracts can be useful to improve the SFE process selectivity. Extraction equipment that operates with three or more separators in series, at different pressures and temperatures, can allow a selective precipitation of different compound families.

Figure 10.4 shows the influence of pressure at temperature of 313 K, and constant mean particle size and flow rate of carbon dioxide on the volatiles isolated from coriander seeds (Grosso et al. 2008). At 9.0 and 10.0 MPa the yield is almost the same and the extract was mainly composed of volatile components, respectively 97 % and 95 %. However, at 15 MPa the yield achieved is higher, but almost 15 % of the extract constitutes of non-volatile compounds not present in essential oil. Although the total amount of recovered oil increased at higher pressures, the selectivity decreased, due to the increase of co-extraction of waxes, fatty acids

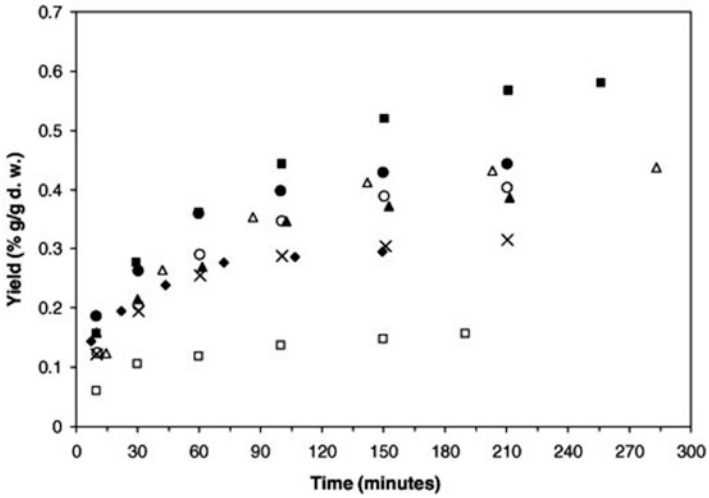


Fig. 10.4 Comparison between the volatile oil yields, from coriander seeds, for different SFE conditions: (*filled triangle*—9 MPa/313 K, *square*—9 MPa/323 K, *circle*—10 MPa/313 K and *filled square*—15 MPa/313 K for a mean particle size of 0.6 mm and a flow rate of 1.1 kg/h; mean particle size (*filled circle*—0.4 mm, *filled triangle*—0.6 mm, ×—0.8 mm) using 9 MPa, 313 K and a flow rate of 1.1 kg/h; and flow rate (*triangle*—0.79 kg/h, *filled triangle*—1.1 kg/h, *filled diamond*—1.56 kg/h) for 9 MPa, 313 K and 0.6 mm of mean particle size. (From Grosso et al. 2008, Fig. 1, published with kind permission of Food Chemistry 2013. All Rights Reserved)

and other heavy components. Moreover, in another study, carried out with the same plant and using extraction conditions of 15 MPa and 323 K (Anitescu et al. 1997), a similar yield was obtained and co-extraction of fatty acids and esters was also observed.

The influence of the temperature using pressure of 9 MPa, 1.10 kg/h and 0.6 mm, can be observed in the same figure. The yield at 313 K was higher than that obtained at 323 K (0.38 vs. 0.16) %, due to the higher density ($\rho = 485$ vs. 285 kg m^{-3}).

The effect of temperature on the solvating power of supercritical fluids is dependent on the solute volatility and solvent density (Reverchon and De Marco 2006). When the temperature increases, at low pressure, a reduction of the solvating influence of the supercritical fluid on the extracts from others plants, such as *Ginkgo biloba* (Chiu et al. 2002), *Artemesia annua* (Xing et al. 2003); *P. nigrum* (Perakis et al. 2005) or *Thymus vulgaris* (Grosso et al. 2010) was found. However, at a higher pressure this effect can change and, for instance, at 25 MPa the rise of temperature increases the extraction rate and the yield of the desirable products. This behavior could be explained by reaching the so-called "cross-over" region.

In $SCCO_2$ extraction of black pepper at 16–26 MPa, temperature of 308–323 K, solvent flow rate from 0.2–0.4 m^3/h, and diameter of ground material of 20–50 meshes the same behavior was found (Zhiyi et al. 2006). In fact, the extraction yield was found to increase with temperature at 26 MPa and to decrease at 16 MPa. This behavior is interpreted observing that at a lower pressure, the change of the solvent density is more efficient than that of the solute vapor pressure, so the extraction rate increases with decreasing of temperature. However, at higher pressures the extraction rate becomes more dependent on the solute vapor pressure, so it increase with temperature.

Pressure and temperature can be used also to improve the quality of the food products, namely, their antioxidant capability. The extraction of vegetable seed oil (SO) and volatile oil (VO), from coriander seeds (Mhemdi et al. 2011) have been performed according to two experimental strategies:

1. Extracting both oils at the same time at a pressure of 21 MPa and temperature of 308 K, fractionating them, and afterwards using two consecutive separators with adequate temperature and pressure conditions. A supercritical fluid flow rate of 1 kg/h and a mean size of 0.5 mm for the ground seeds are the best conditions for SFE. This study also shows that at 7 MPa and 303 K in the first separator and 4 MPa and 283 K in the second one allows to recover the SO and VO, respectively.
2. Extraction of both oils in a separate way using two consecutive extraction steps. The first extraction step is performed at 8 MPa and 308 K in order to extract only the VO. Then, 21 MPa and 313 K are the convenient extraction conditions for the second step which allows extracting SO.

The first strategy has been applied with success in the SFE of volatile oils (Reverchon 1992, 1997; Reverchon et al. 1995, 1999).

Ibáñez et al. (1999) applied the second approach to the fractionation of rosemary extract with two successive extraction steps, resulting in a volatile oil rich fraction

with a low antioxidant activity in the first step (10 MPa and 313 K) and a high-antioxidant activity fraction in the second one (40 MPa and 333 K).

The same strategy was used in SFE of compounds from *Satureja montana* (Grosso et al. 2009b), as mentioned in Sect. 10.4. Furthermore, an identical approach has been followed to the extraction of volatiles and no-volatiles from *Thymus vulgaris* (Grosso et al. 2010).

10.5.3.2 Supercritical Fluid Flow Rate

The supercritical fluid flow rate through the extractors has a strong influence on the extraction efficiency. Solvent flow rate can also be expressed by the linear velocity, which is strongly dependent on the flow rate and the extractor geometry. For a given system, the flow rate can be easily changed by using extractors with different inside diameters.

If it is assumed that the supercritical fluid can penetrate deeper in the matrix, the decreasing of flow rate causes a lower linear velocity and increases the extraction of solute (solubility is the limiting factor).

Otherwise at this condition, a higher flow rate can help to reduce partitioning back onto matrix sites. Larger particles decrease the packing density. On the other hand, a smaller particle size and larger extractors reduce potential matrix effects. These considerations, together with the sample particle size and extractor packing density uniformity, can be important factors in SFE processes (Bowadt and Hawthorne 1995).

In some of the systems investigated it was shown that the increase of the flow rate benefited the extraction rate. On the contrary, other authors concluded that the yield decreased with rise of the flow rate of the solvent. However, typically the solvent flow rate did not affect the global composition of the $SCCO_2$ extracts.

Examples of negligible or small dependence of the flow rate in the extraction process can be found in the literature (Roy et al. 1996; Zekovic et al. 2001). In the extraction of ginger oil it was verified that the extraction curves were independent of flow rate in a plot of oil yield versus extraction time (Roy et al. 1996), indicating that the extraction process is controlled by intraparticle diffusion within a particle of ginger root. However, the extraction rate increased, as the particle size decreased, due to a decrease in the diffusion path. In addition the same behavior was verified for the system thyme oil–CO_2 (Zekovic et al. 2001).

A negative effect on the extraction yield due to the increase of the flow rate of the solvent has been described (Papamichail et al. 2000; Louli et al. 2004b). In the system celery seed oil–CO_2 (Papamichail et al. 2000), the amount of the extracted oil per kg of CO_2 used is higher for the lower flow rate, which the authors attributed to the intraparticle diffusion resistance.

In Fig. 10.4 the negative influence on the volatile oil yield from coriander seeds with the rise of flow rate is shown (Grosso et al. 2008). However, this behavior had no influence in the global composition of the volatile oil.

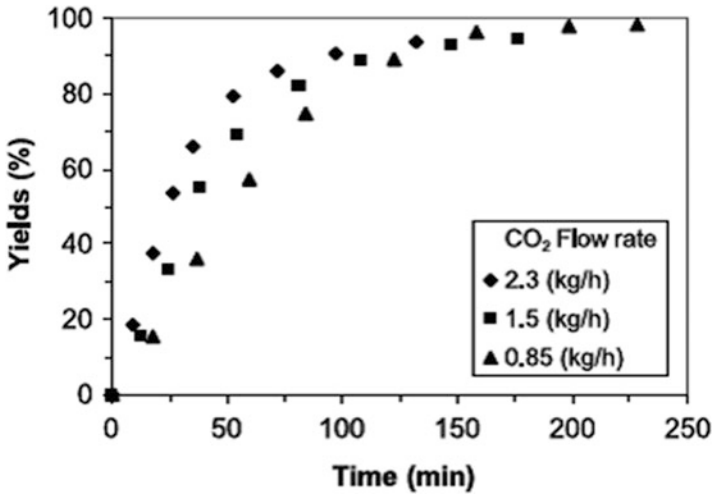


Fig. 10.5 Percentage of total oil (yield) from fennel for 0.55 mm particles, at different CO_2 flow rates as a function of time, at the selected condition of extraction and separation. (From Coelho et al. 2003, Fig. 1, published with kind permission of Flavour and Fragrance Journal 2013. All Rights Reserved)

A positive effect on the yield of extract as the flow rate increases can be found more frequently in the literature. In the extraction of turmeric oil with supercritical carbon dioxide in a semi continuous-flow extractor, it was reported that the extraction rate increased with the CO_2 flow rate (Gopalan et al. 2000). The same behavior occurs in the extraction of volatile oil from fennel, as shown in Fig. 10.5.

Moreover, $SCCO_2$ extraction of black pepper at 16–26 MPa, temperature of 308–323 K, with a solvent flow rate from 0.2 to 0.4 m^3/h, and diameter of ground material of 20–50 meshes was investigated, and the optimum process condition of the SFE for pepper oil was determined, suggesting that the extraction rate increases also with a rise in the flow rate of carbon dioxide from 0.2 to 0.4 m^3/h (Zhiyi et al. 2006).

10.5.3.3 Modifiers

Supercritical carbon dioxide, a "green" solvent, has a polarity, dielectric constant and dipole moment lower than those of most conventional organic solvents, which can be a serious problem for extraction of polar compounds. In fact, CO_2 is an illustration of a simple, non-dipolar solvent system. Although CO_2 has a zero dipole moment, it is a charge-separated molecule, with significant nonzero bond dipole moments. This charge separation determines a significant quadrupole moment and, therefore, CO_2 is a quadrupolar solvent.

A more "chemical" description has evolved recently regarding the solvation behavior of CO_2, which can act as a weak Lewis acid (LA), as well as a Lewis base (LB). As a consequence, CO_2 can solubilize several dipolar and non-dipolar molecular systems facilitated by site-specific solute-solvent interactions (Raveendran et al. 2005).

Carbon dioxide as a solvent requires the addition of a co-solvent, also called modifier or entrainer, particularly to extract more polar compounds (such as phenolic antioxidants). Co-solvents, like ethanol or isopropanol, are polar compounds that, added in small amounts, can change substantially the solvent properties of the supercritical CO_2 (Herrero et al. 2006; Reverchon and De Marco 2006).

The use of modifiers increases the range of materials which can be extracted. The most common co-solvents are short chain alcohols, among which ethanol and methanol predominate. These alcohols show ability for inducing dipole/dipole interactions and hydrogen bonding with polar functional groups. Usually, they are added in a percentage that changes from 1 to 15 % (Zarena et al. 2012; Kagliwal et al. 2012).

Modifiers, such as ethanol are often used, which improves the collection of the extracted material. However, some of the benefits of using a solvent (CO_2) which is gaseous at room temperature are reduced. In fact, since the modifier is in the liquid phase at atmospheric pressure, it will be collected in the separator together with the extracted compound and, therefore, a post-processing evaporation of the co-solvent is necessary (Rodrigues et al. 2003).

Generally, the selection of the SFE conditions (temperature, pressure, and percentage of cosolvent) will depend on the intended use of the extract. The presence of toxic residues implies that their lower limits allowed in the extracts should be considered. The use of a suitable cosolvent may increase the performance and the economic feasibility of a given process, improving the extraction yield and separation selectivity.

Sage herb (*Salvia officinalis* L.) was submitted to extraction at 25 and 35 MPa pressure and 313 K with carbon dioxide and the extracts tested on their antioxidant activity (Dauksas et al. 2001). SFE of sage at a pressure of 35 MPa was found to be an effective method to obtain pure extracts. The yields of the extracts were substantially further increased (almost 2–4 times) by using 1–2 % of entrainer solvent ethanol. However, the fractionation of sage extract (in three separators process) was a complex procedure in terms of extract distribution between separators operating at various pressure and temperature conditions.

Hamburger et al. (2004) performed extractions on chamomile (*Matricaria recutita*), marigold (*Calendula officinalis*) and hawthorn with $SCCO_2$ with mixtures of supercritical carbon dioxide plus ethanol of varying proportions (0–20 % ethanol) and at various pressures in the range 30.0–68.9 MPa.

The authors concluded that the extraction yield under several conditions depends to a large extent on the profiles of the secondary metabolites present in the three plants. For instances to marigold flowers the extractability of lipophilic compounds increased substantially at pressures above 30.0 MPa (Fig. 10.6), the yields of polyphenolic and glycosidic compounds remained low even at 68.9 MPa and with 20 % modifier in the extraction fluid.

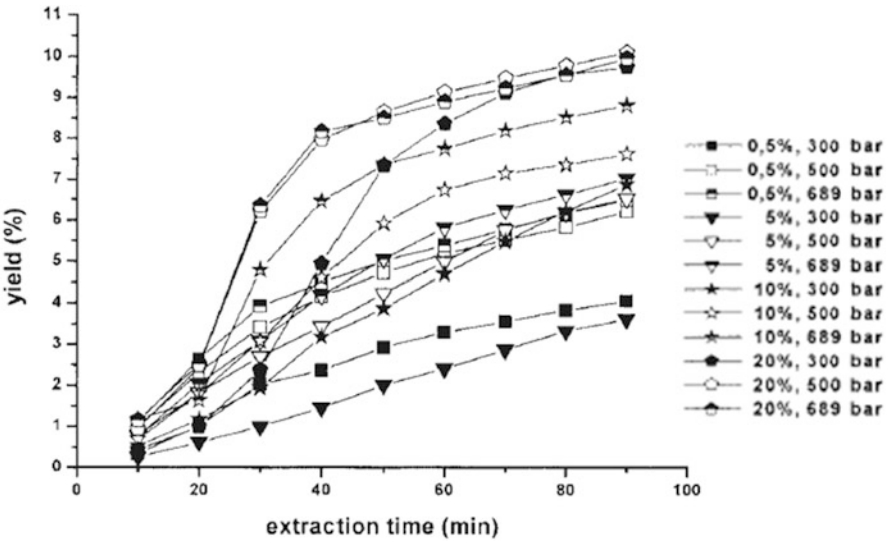


Fig. 10.6 Extraction profiles representing yields (%w/w) of extractable obtained from marigold flowers, extracted with carbon dioxide: ethanol mixtures containing 0.5, 5, 10, 20 % modifier at pressures between 30.0 and 68.9 MPa (each curve shown was constructed from the results of triplicate experiments). (From Hamburguer et al. 2004, Fig. 3B, Published with kind permission of Phytochemical Analysis 2013. All Rights Reserved)

Functional (antioxidant, antimycobacterial, and anticancer activities) properties of spice extracts from ginger (Zingiber *officinale* R.), rosemary (*Rosmarinus officinalis* L.) and turmeric (*Curcuma longa* L.) obtained using $SCCO_2$ with/without modifier (ethanol and/or isopropyl alcohol) were carried out at pressures between 10.0 and 30.0 MPa and temperatures between 303 and 313 K (Leal et al. 2003). The authors showed that the antioxidant activities of the SFE extracts were superior to the activity of β-carotene; e.g. the rosemary extracts exhibited the strongest antioxidant activity and an anti-mycobacterial activity 4–8 times higher than that of turmeric and ginger extracts. However, ginger and turmeric extracts showed selective anticancer activities.

Several co-solvents can be used and tested to find the best yields or the less toxic effect, which sometimes are not dissociated. For instance, a study on the effect of different modifiers (e.g., methanol, ethanol, dichloromethane, and hexane) on the volatile oil extractions from *Perovskia atriplicifolia* at constant pressure and temperature (Pourmortazavi et al. 2003) showed that hexane was more selective than the other modifiers when the extraction of the plant was carried out at low pressure (10.0 MPa) and temperature (308 K).

Moreover, mixtures with higher content of co-solvent can be used to improve the extraction of compounds from spice matrices. Thus, turmeric extracts rich in curcuminoids were obtained in a fixed bed extractor at 30.0 MPa, 303 K using a mixture of CO_2 and ethanol/. The SFE using 50 % of the cosolvent employed during the static period increased the curcuminoid content (0.72 % of curcuminoids) and reached approximately 10 % of extract yield (Braga and Meireles 2007).

10.6 Spices and Herbal SFE Extracts

The progress of industrial applications of SFE, particularly important in the field of nutraceuticals and pharmaceutical products, are followed with attention by various international companies. Studies were carried out on the production of SFE extracts from some of the most important spices and herbs, such as black pepper, clove, coriander, Capsicum species, fennel and thyme.

10.6.1 Black Pepper

The content of essential oil from black pepper is 1.5–6.2 % (w/w) and it consists mainly of monoterpenes and sesquiterpenes. However, the total content in oleoresin can achieve values up to 13 % where the presence of piperine is of primordial importance. The SFE and fractionation of black pepper was studied systematically taking into account the effects of operating parameters, like extraction pressure, temperature, flow rate of CO_2 and the particle size of the raw material, on the extraction rate and yield.

Table 10.7 presents studies on SFE of compounds from black pepper.

Assumptions that a plug flow of the solvent exists in the fixed bed of solid particles and that axial dispersion is negligible (Sovová et al. 1995), conclude that

Table 10.7 Operating parameters in SFE and global yield of extraction of compounds from black pepper

Process parameters/observations	Global yield of extraction (%)	Reference
P = 28 MPa; T = 297–333 K; F = 0.8–1.9 g/min^{-1} d_p = 0.05 mm; W (w/w) = 11.5 %	OR = 6.7–7.6 (50–54 % piperine)	Sovová et al. (1995)
P = 8–10 MPa; T = 313–333 K; P = 20–32 MPa; T = 318–338 K; d_p = 40 mesh	VO = 0.71; OR = 4.6–6.47 (32.2–39.4 % piperine)	Tipsrisukond et al. (1998)
P = 15–30 MPa; T = 303–323 K; F = 0.14–0.67 g/min^{-1}; d_p = 60 mesh	O = 0.49–2.05	Ferreira et al. (1999)
P = 9–15 MPa; T = 313–323 K; F = 1.0–3.0 kg/h; d_p = 0.175 mm; W (w/w) = 9 % (fractional separation, two vessels)	OR = 13.2	Perakis et al. (2005)
P = 16–26 MPa; T = 308–323 K; F = 0.2–0.4 m^3/h; d_p = 20–50 mesh (fractional separation, two vessels)	O = 6.5	Zhiyi et al. (2006)
P = 20–30 MPa; T = 313–333 K; F = 3 mL/min		Topal et al. (2008)
P = 7.5–15 MPa; T = 303–323 K; d_p = 0.25–0.50 mm; W (w/w) = 11 %	OR = 9.3	Kumoro et al. (2010)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. *O* oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content

the rate of extraction increases with temperature, because of the solubility of piperine in carbon dioxide rises, increase at the first period of extraction while in the second extraction period also due to the faster diffusion of all components through the plant tissue. In the best conditions it is possible to obtain yields 7.6 % of oil with a content of 54 % of piperine.

Other authors, e.g. Tipsrisukond et al. (1998), obtained similar yields of recovery as solvent-extracted (6.1 %) with the one obtained with $SCCO_2$ at pressures of 28 MPa and 55 °C (6.13 %). These results are in agreement with the optimal process condition of the SFE for pepper oil at 22–26 MPa, 318 K, found by Zhiyi et al. (2006).

These optimal conditions are associated with solvent density and change in solute vapor pressure. At lower pressure, the change of solvent density is more effective than that of the solute vapor pressure, as extraction rate increases with a decrease in temperature. However, the increase in solvent density with pressure overcomes the relatively small change of solute vapor pressure; for instance, at 25 MPa the extraction rate is dependent on the solute vapor pressure increasing with temperature (Zhiyi et al. 2006). The retrograde condition was found at 15 MPa and also the increase of particle size reduced the extract yield and extraction rate (Kumoro et al. 2010).

$SCCO_2$ volatile oils were found to have greater concentrations of sesquiterpene and oxygenated hydrocarbons than EO obtained by conventional method (Tipsrisukond et al. 1998; Kumoro et al. 2010). The best condition to obtain the greatest relative extraction rate of VO compounds by extraction with $SCCO_2$ was found at 10 MPa and 313 K (Tipsrisukond et al. 1998). It was verified that the amount of extracted VO increased with the CO_2 density and that the extraction yield was significantly enhanced as pressure increased or temperature decreased. Furthermore, the extraction rate the with the flow rate (Perakis et al. 2005).

10.6.2 Capsicum

Chillies (*Capsicum frutescens*), paprika (*Capsicum annuum*) or others *capsicum* genus and species contain oily, aromatic, pungent and color compounds. This oleoresin is a lipidic extract, red and sticky oil, with a characteristic flavor. The interest in this oleoresin is mainly due to its colorant power. This property has a number of applications in food, cosmetic, and pharmaceutical industries (Sovilj et al. 2011). Moreover, it represents one of the first spice raw material (import/export) in the world (please see Sect. 10.7).

In Table 10.8 are presented studies of SFE of the capsicum genus and species, which shows that the total oleoresin yield and the principal capsaicinoids content increase with the pressure at constant temperature since the solvent power of $SCCO_2$ increases. Moreover, this increase in solubility of the compounds is mainly due to a large driving force for mass transfer at high pressure than lower pressure (Uquiche et al. 2004).

The particle size is another important parameter in the process. In fact, it was observed that as average diameter decreases, the extraction rate increases.

Table 10.8 Operating parameters in SFE and global yield of extraction of compounds from *Capsicum* genus and species

Process parameters/observations	Global yield of extraction (%)	Reference
P = 10–40 MPa; T = 308–328 K; F = 1.0–1.5 L/min; d_p = 0.2 mm;	OR = 3–7.2	Illés et al. (1999)
Also sub-critical propane (P = 8 MPa; T = 298–323 K)	Propane: OR = 8.8	
P = 10–40 MPa; T = 308–328 K; F = 1.0–1.5 L/min; d_p = 0.4–0.6 mm; W (w/w) = 10–11 %	OR = 4–11.5	Daood et al. (2002)
Also sub-critical propane (P = 8 MPa; T = 298–323 K)	Propane: OR = 11–11.5	
P = 32–54 MPa; T = 313 K; d_p = 0.23–3.90 mm; U = 0.57–1.25 mm/s; W (w/w) = 4.4 %	OR = 4–5.8	Uquiche et al. (2004)
P = 16.2–23 MPa; T = 313 K; d_p = 0.27 mm; U = 0.064–0.074 cm/s	OR = 7.8–9.6	Duarte et al. (2004)
P = 15–35 MPa; T = 313–333 K; F = 1.98×10^{-4} kg/s; d_p = 0.5–0.8 mm; W (w/w) = 6–8 %	OR = 11.8–13.4	Aguiar et al. (2013)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. *O* oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content, *U* linear velocity

The results were explained assuming spherical particle geometry and the dimensionless Biot number, *Bi*, which gives the relative importance of intraparticle diffusion and external solid-SCF mass transfer. The total particle surface per unit volume increases from 750 to 10,990 m^{-1} as average particle size decreases from 3.90 mm (*Bi* = 170) to 0.273 mm (*Bi* = 19) and there was a shift in the controlling resistance from intraparticle diffusion and external solid-SCF mass transfer (Uquiche et al. 2004).

Phase equilibrium of oleoresin *Capsicum* in $SCCO_2$ was measured by a recirculation method at pressures between 9.0 and 31.5 MPa and temperatures of 314, 326 and 338 K (Fernandez-Ronco et al. 2011). The authors justified the studies carried out with the great interest in compounds, such as carotenoids and capsaicinoids, and in how the enrichment processes to transform this natural food colorant into other products with application in pharmaceutical or cosmetic industry, besides food industry.

10.6.3 Clove

Clove oil has been used widely as pharmaceutical, flavoring and antimicrobial agent in food industry. In Table 10.9 studies on supercritical extraction of compounds from spice clove are presented.

Using a fractional separation technique in a range of pressure of 8–20 MPa, temperature of 313–323 K, mean particle size of 0.35 mm, and CO_2 flow rates

Table 10.9 Operating parameters in SFE and global yield of extraction of compounds from spice clove

Process parameters/observations	Global yield of extraction (%)	Reference
P = 9 MPa; T = 323 K; F = 0.6–1.2 kg/h; d_p = 0.37 mm; (fractional separation, two vessels)	VO = 20.8	Reverchon and Marrone (1997)
P = 8–20 MPa; T = 313–323 K; d_p = 0.37 mm; F = 0.6–1.2 kg /h (fractional separation, two vessels)	VO = 20.7	Della Porta et al. (1998)
P = 20 MPa; T = 328 K (also with SWE)	OR = 18.34–21.54	Clifford et al. (1999)
P = 10–30 MPa; T = 303–323 K; F = 2 L/min; d_p = 0.52–0.79 mm	VO = 18.3–23.95	Wenqiang et al. (2007)
P = 12 MPa; T = 318 K	VO = 21.04	HongPeng et al. (2009)
P = 10 MPa; T = 313 K; F = 0.62 kg /h; d_p = 0.4 mm; W (w/w) = 8.78 %	VO = 18.2	Ivanovic et al. (2011)
P = 10 MPa; T = 313 K; F = 0.62 kg/h; d_p = 0.4 mm; W (w/w) = 8.78 %	VO = 18.2	Ivanovic et al. (2013)
P = 25–50 MPa; T = 313–403 K; F = 2 mL/min; d_p = 0.5 mm	*Yield of eugenol* (54–132 mg g^{-1})	Chatterjee and Bhattacharjee (2013)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. *O* oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content, *SWE* superheated water extraction

between 0.6 and 1.2 kg/h it is possible to isolated volatile oil from clove bud (Della Porta et al. 1998). The preliminary studies indicated that 9 MPa and 323 K were the best extraction conditions, because no undesired compounds were found.

Other authors have compared the oil obtained using SFE with that from hydrodistillation, steam distillation and Soxhlet extraction (Wenqiang et al. 2007) concluding that at pressure of 10 MPa and temperature of 323 K, a 19.56 % (w/w) yield of volatile oil can be obtained from clove buds; thus SFE proved to be the optimum process among the four processes examined for obtaining clove oil with high quality.

In 2011 and 2013 another strategy was advocated (Ivanovic et al. 2011, 2013). A mixture of two plants was used with the aim to explore and explain kinetics and mass transfer phenomena during the SFE process. Therefore, mixtures of clove/oregano and clove/thyme with different initial composition were processed by SFE. It was demonstrated that the presence of a small amount of oregano and thyme in the starting mixture with clove bud enables a substantial increase of the extraction rate at the beginning of the extraction process. The explanation for this behaviour was that the lighter compounds from oregano or thyme act as modifier or co-solvent and thus change the $SCCO_2$ solubility power. This effect causes an increase of the solubility of heavier and less soluble compounds present mainly in clove buds. Consequently, addition of a small amount of oregano or thyme allows to reach a desirable extraction yield (up to 90 % of the extraction yield from the pure clove) with 70 % lower consumption of the $SCCO_2$, which could be important for industrial-scale application.

10.6.4 Coriander

Coriander (*Coriandrum sativum* L.) is an annual Apiaceae (*Umbelliferae*) herb which grows in the Mediterranean countries and is widely used in food and pharmaceutical industries (Grosso et al. 2008).

By steam distillation or hydrodistillation only (0.5–2.0) % (w/w) of essential oil can be recovered from the coriander seed. However, volatile oil can be extracted with a substantial increase of the yield (19 %) as presented in Table 10.10.

Three studies report the $SCCO_2$ extraction and recovery of compounds in a two-stage separation system (Anitescu et al. 1997; Grosso et al. 2008; Mhemdi et al. 2011). The results from SFE were compared with those from hydrodistillation in the case of essential oil/volatile oil and with Soxhlet in the case of oil obtained at pressures higher than 15 MPa.

Propane was used as an alternative solvent to CO_2 extraction in the case of coriander seed extraction under sub- and supercritical conditions (Illés et al. 2000). The ratio of solvent to seed to achieve a complete oil extraction at pressures of 20.0 and 30.0 MPa and temperature of 308 K was between 20 and 40 g/g. A complete oil recovery could be attained with propane or propane-rich solvents at 298 K and 5.0, 8.0 and 10.0 MPa. The extract consists of, besides essential oils, triglycerides (glycerol triesters of fatty acids) and waxes. Studies of the antioxidant properties of the volatile oil from coriander were performed and it was demonstrated that it poses a more significant activity, compared to that of commercial antioxidants (Yepez et al. 2002). These studies suggested that supercritical extraction is a promising processing alternative for producing odorless and tasteless antioxidant fractions from coriander seeds.

Table 10.10 Operating parameters in SFE and global yield of extraction of compounds from coriander

Process parameters/observations	Global yield of extraction (%)	Reference
P = 15 MPa; T = 323 K; F = 50 mL/min; d_p = 0.4 mm (fractional separation, two vessels)	VO = 0.61	Anitescu et al. (1997)
P = 10–35 MPa; T = 308 K; subcritical(T = 298 K) subcritical propane—*P* = 5–8 MPa; *T* = 298 K; d_p = 20 mesh; mixtures: propane + CO_2	O = 15.3–16.9	Illés et al. (2000)
P = 11.6–28 MPa; T = 311–331 K; F = 0.08 kg/h; d_p = 1.015 mm; W (w/w) = 5 %	VO = 0.880–1.849	Yepez et al. (2002)
P = 9–15 MPa; T = 313–323 K; F = 0.79–1.56 kg/h; d_p = 0.4–0.8 mm; W(w/w) = 8.5 % (fractional separation, two vessels)	VO = 0.06–0.58	Grosso et al. (2008)
P = 5–10 MPa; T = 298–308 K; F = 2 L/min	VO = 0.19–0.33	Chen et al. (2009)
P = 10–21 MPa; T = 308–328 K; d_p = 0.3–0.9 mm; F = 1–2 kg/h; W (w/w) = 10 %; (fractional separation, two vessels)	VO = 0.36 O = 18.72	Mhemdi et al. (2011)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. *O* oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content

10.6.5 Fennel

Fennel is a perennial herbaceous plant which grows in good soils in sunny mild climatic regions. The leaves, stalks and fruits of the plant are edible. The essential oil or oleoresins from fennel seeds are important ingredients for flavoring cosmetics, pharmaceuticals, and food products; hence, the improvement of the quality of fennel oil is of economic importance (Simandi et al. 1999). In essential oil, trans-anethole and fenchone are the most important volatile components as well as in the non-volatile extracts where together with stearic, oleic and linoleic acids are the more important compounds (Moura et al. 2005).

Table 10.11 presents studies on SFE of compounds from fennel, with and without fractional separation, which allowed obtaining better volatile compounds (without waxes or fatty acids).

$SCCO_2$ extraction of compounds from fennel seeds has been achieved in two steps: in the first step (9 MPa and 323 K) selective extraction of volatile oil is achieved, while the second (20 MPa and 313 K) allows the extraction of vegetable oil (Reverchon et al. 1999). The experiments were performed using the fractional separation technique to obtained extracts using three different CO_2 flow rates 0.5, 1.0, and 1.5 kg/h.

Moreover, subcritical water extractions (SWE) in a continuous method for the isolation of the essential oil of fennel have been carried out (Gámiz-Gracia and Duque de Castro 2000). The principal advantages of the method against traditional methods are the shorter extraction times (50 min against 4 h for hydrodistillation and 24 for manual dichloromethane extraction) and the lower cost of energy.

Table 10.11 Operating parameters in SFE and global yield of extraction of compounds from fennel

Process parameters/observations	Global yield of extraction (%)	Reference
P = 9–20 MPa; T = 313–323 K; F = 0.5–1.5 kg/h; d_p = 0.37 mm (fractional separation, two vessels)	VO = 1.8 O = 9.2	Reverchon et al. (1999)
P = 30 MPa; T = 313 K; d_p ~ 0.8 mm; W (w/w) = 10.8 % (fractional separation, two vessels)	VO = 0.69–4.27 O = 5.82–9.88	Simandi et al. (1999)
P = 20–35 MPa; T = 318–328 K (with 5 % methanol)		Yamini et al. (2002)
P = 9–10 MPa; T = 303–313 K; F = 0.85–2.3 kg/h; d_p = 0.35–0.75 mm; W (w/w) = 7.5 % (fractional separation, two vessels)	VO = 3.0	Coelho et al. (2003)
P = 10–30 MPa; T = 313–323 K; F = 8.33×10^{-5} kg s^{-1}; d_p = 0.61 mm; W (w/w) = 7.5 %	O = 3.1–12.5	Moura et al. (2005)
P = 8–15 MPa; *T* = 313–330 K; d_p = 0.9 mm; F = 0.2-5.7 kg/h; W(w/w) = 9.6 %	VO = 1.5–5.2	Damjanovic et al. (2005)
P = 20–30 MPa; T = 313–333 K; F = 3 mL/min		Topal et al. (2008)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. O oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content

10.6.6 Thyme

Thyme (*Thymus vulgaris*) is a common ingredient in cooking and is used also in herbal medicine. The strongly antiseptic and antifungal activities of thyme essential oil are mainly due to the presence of phenolic compounds, such as thymol and carvacrol (Zeković et al. 2000).

SFE of the volatile oil from *T. vulgaris* aerial flowering parts was performed under different conditions of pressure, temperature, mean particle size and CO_2 flow rate.

In Table 10.12 are presented studies of SFE of compounds from thyme.

A high pressure apparatus equipped with 5 L volume extractor vessel was used for the SFE at pressure up to 40 MPa and temperature 333 K. The result obtained were compared with those attained by Soxhlet alcoholic extraction (Simandi et al. 2001). Using two different methods for measuring oxidative stability, it has been confirmed that both alcoholic and supercritical CO_2 extracts of thyme show substantial antioxidant activity.

On the other hand, in a pilot-plant, the SFE of compounds from thyme was carried out at 313 K and at different extraction pressures of 15, 30 and 40 MPa (García-Risco et al. 2011). The concentration of terpenoid-type compounds (thymol, carvacrol, borneol, etc.) in the extract was higher at the lower extraction pressure employed.

Table 10.12 Operating parameters in SFE and global yield of extraction of compounds from thyme

Process parameters/observations	Global yield of extraction (%)	Reference
P = 8–40 MPa; T = 313 K; F = 97.73 L/h; d_p = 0.35 mm (fractional separation, one vessel)	VO = 0.48	Zeković et al. (2000)
P = 8–20 MPa; *T* = 300–323 K; W (w/w) = 10–12 %	OR = 6–12	Moldão-Martins et al. (2000)
P = 40 MPa; T = 333 K; d_p = 0.2–0.8 mm; W (w/w) = 9 % (fractional separation, two vessels)	OR = 4.92	Simandi et al. (2001)
P = 12 MPa; T = 313 K; W (w/w) = 9.9 %		Maroto et al. (2005)
P = 11.5 and 35 MPa; T = 313 and 373 K; d_p = 0.3 mm; F = 0.3 kg/h	OR = 1.58	Babovic et al. (2010)
P = 9–25 MPa; T = 313–323 K; d_p = 0.4–0.8 mm; F = 0.7–1.3 kg/h; W(w/w) = 10.9 % (fractional separation, two vessels)	VO = 0.4–1.1	Grosso et al. 2010
P = 11.5 and 35 MPa; *T* = 313 and 373 K; d_p = 0.4 mm; F = 0.3 kg/h	EO = 1.23	Ivanovic et al. (2011)
P = 15–40 MPa; T = 313 K; d_p = 0.2–0.6 mm; F = 40 g/min (fractional separation, two vessels)	E = 3.3–4.7	García-Risco et al. (2011)
P = 35 MPa; T = 373 K		Rodrigues et al. (2013)

The nomenclature of the authors is adopted in all cases, except when it is clear that volatile oil (VO) is obtained. *O* Oil, *OR* Oleoresin, *E* extract, *F* flow rate, d_p particle size, *W* moisture content

Three different fractions from the original supercritical extract were obtained when the supercritical extract was fractionated. Around a two fold increase of thymol content was achieved at 15 MPa, 323 K and 3 % ethanol co-solvent.

10.7 Economic Feasibility and Market Trends

The most important spice crops from the tropical regions are pepper, capsicums, nutmeg/mace, cardamom, allspice/pimento, vanilla, cloves, ginger, cinnamon, cassia, and turmeric. On the other hand, coriander, cumin, mustard, sesame seeds and sage, oregano, thyme, bay and the mints are the most important spice crops from the temperate zone.

In terms of world trade, the value of global spice imports is estimated at 2–2.4 billion US dollars in 2002. Pepper is at the top of the list with 20 % of the total value followed by capsicum (18 %), vanilla (13 %), nutmeg/mace/cardamom (9 %), spice seeds (8 %) and ginger (6 %). The major spice production is in the tropics, in developing and least developed countries (Douglas et al. 2005).

In the European Union, Germany, followed by the Netherlands, United Kingdom and Spain are the largest spices and herbs importers. As exporter, the Netherlands, followed by Spain, Germany and Bulgaria, are the leader. Leading spices and herbs suppliers to the European Union-27 (EU-27) in 2012 have been China (19 %), India (10 %), Vietnam (7 %), Indonesia (4.4 %), Peru (4.1 %) and Brazil (3.3 %). However, based on the value of imports, Vietnam and Indonesia are the largest exporters. This can be explained by the fact that the average price of spices and herbs exported by Vietnam (€5.7/kg) and Indonesia (€6.2/kg) is much higher than of that from China (€1.5/kg). The progress of excellence of the species can be an opportunity to high value markets.

The United States of America continues to be the worldwide leading consumer of dried capsicum or pimenta crushed or ground, followed by the EU-27. While over 90 % of USA imports of chilies come from Mexico, Caribbean countries continue to be a major supplier of the hottest peppers imported by the USA. Canada also imports hot peppers from the Caribbean but in a smaller scale. Exports to Canada usually land in cities where there are high populations of Asians and West Indians (Toronto and Montreal), who are significant consumers of hot peppers.

These values can be updated by accessing to the Commodity Trade Statistics DatabaselUnited Nations Statistics Division, where the data corresponding to the different countries or areas are regularly renovated. In Tables 10.13 and 10.14 the export and import values of the European Union-27, United States of America and the rest of the world are presented.

The importance of the EU-27 and USA as importers of spices and herbs (Table 10.14), as contrasted to their low export, should be noted.

The top three exporting and importing individual countries and the respective spices/herbs types quantities from 2010 to 2012 are presented in Tables 10.15 and 10.16, respectively. China, with the export of ginger, appears as the principal world exporter, followed by India with capsicum or pimenta dried crushed or ground in

Table 10.13 Export of spices and herbs for the period 2010–2012, quantities in tons; trade in 1,000 USD, to European Union-27 (EU-27), United States of America (USA) and the rest of the world

	Quantity (ton)			Trade (×1,000 USD)		
	2010	2011	2012	2010	2011	2012
EU-27	83,560	87,280		319,940.1	313,748.4	
USA	25,490	28,150	31,760	100,055.7	118,091.0	135,280.9
Rest of the World	1,567,180	1,887,410	2,038,150	4,221,285.9	5,572,488.3	4,796,913.5

Source: Commodity Trade Statistics DatabaseUNST, access in December 2013 http://unstats.un.org/unsd/default.htm, http://comtrade.un.org/db/

Table 10.14 Import of spices and herbs for the period 2010–2012, quantities in tons; trade in 1,000 USD, to European Union-27 (EU-27), Unite States of America (USA) and the rest of the world

	Quantity (ton)			Trade (×1,000 USD)		
	2010	2011	2012	2010	2011	2012
EU_27	273,755	287,068	2,909,893	950,560.7	1,161,908.1	1,169,363.9
USA	300,259	206,403	326.424	843,125.5	814,556.1	1,159,801.4
Rest of the World	1,380,720	1,303,320	1,159.92	2,631,125.9	3,631,330.1	2,691,644.7

Source: Commodity Trade Statistics Database | UNST, access in December 2013 http://unstats.un.org/unsd/default.htm, http://comtrade.un.org/db/

Table 10.15 Top three exporting individual countries (non EU-27) by quantity for the period 2010–2012, trade and spice or herb type quantity

Country	Year	Spice or herb	Trade (USD)	Quantity (ton)
China	2012	Ginger	261,729,300	448,070
India		Capsicum or pimenta dried crushed or ground	532,037,400	369,280
India		Others spices	123,316,900	118,100
China	2011	Ginger	409,484,200	408,850
India		Capsicum or pimenta dried crushed or ground	496,068,800	260,490
India		Others spices	125,430,100	106,250
China	2010	Ginger	434,604,700	299,850
India		Capsicum or pimenta dried crushed or ground	347,901,900	118,540
India		Turmeric (curcuma)	145,325,400	107,920

that period. USA and Japan are the principal importing countries of capsicum or pimenta dried and ginger, respectively.

In 2012, other spices attained great importance in the world trade market. For instance, the export of Turmeric (curcuma) from India (98,700 ton), cumin seeds from India (90,000 ton), and pepper of the genus *Piper* from Indonesia

Table 10.16 Top three importing individual countries (non EU-27) by quantity for the period 2010–2012, trade and spice or herb type quantity

Country	Year	Spice or herb	Trade (USD)	Quantity (ton)
USA	2012	Capsicum or pimenta dried crushed or ground	304,781,900	120,460
Japan		Ginger	102,832,000	71,720
Malaysia		Capsicum or pimenta dried crushed or ground	101,375,800	56,300
USA	2011	Capsicum or pimenta dried crushed or ground	278,490,100	109,940
Japan		Ginger	123,666,300	65,460
Pakistan		Ginger	51,014,700	60,110
USA	2010	Capsicum or pimenta dried crushed or ground	223,521,400	104,570
Nigeria		Nutmeg	1,268,655	85,570
Malaysia		Capsicum or pimenta dried crushed or ground	123,496,800	75,220
Japan		*Ginger*	*96,794,750*	*65,370*

(61,600 ton), shows the significant capacity of those spices and countries as major players in the world trade market.

Moreover, if the analyses of the data available in the Commodity Trade Statistics DatabaselUNST were performed by trade market (USD) in the same year, the leading exported spice is capsicum or pimenta dried crushed or ground from India (532,037,360 USD), Indonesia (416,319,370 USD) and China (263,718,170 USD). Moreover, Indonesia and Singapore with cloves (whole fruit cloves and stems) 110,792,870 and 106,596,470 USD, respectively, and India with pepper of the genus piper whole (106,540,210 USD) are the leader importer.

Volatile oils, spices oleoresins as well as soft extracts from black pepper, cumin fennel, ginger, nutmeg, clove, cardamom, vanilla, capsicum, celery or nutmeg, are produced by SFE in countries like Germany, France, USA, India and China, for example. The industrial applications of SFE to spices and herbs are a reality in these countries and can be readily found in the market.

Spices, herbs and essential oil crops are produced intensively by industry mainly in the tropics, providing export opportunities for developing countries. Additionally, the importance in organic spices, herbs and essential oils continues to grow along with the overall market for organic food and beverages. Industry cost differentiates among developing countries and target markets and demand for tropical and arid climate products provide value adding opportunities for developing countries in exporting finished products. Generally, these products will need to be promoted in co-operation with marketing companies and under varieties recognized in the importing markets—just as for other processed or packaged products (Jack 2006).

As value adding requires investments of money, skills and time, the immediate marketing opportunities are currently increasing the supply of bulk spices, herbs and essential oils.

A clean labeling for food ingredients and additives that are organic/natural with names that are familiar, and that are perceived to be healthy are in a clear demand from the consumers. In addition, the call for sustainable sources and

environmentally friendly production is compelling the food industry to move in that direction (Berger 2009).

Producers should seek to offer foodstuffs that have organic certification, with high quality and that can be easily supplied. They should also endeavor to improve their marketing control and overall company/product profile by offering their customers additional quality certifications, such as Hazard Analysis Critical Control Point HACCP, European Retailers Produce Working Group—Good Agricultural Practices, EurepGAP, and International Organization for Standardization, ISO. In this point, volatile oils, oleoresins or extracts from spices and herbs without toxic solvents or residues will play an important role.

Moreover, taking into account the advantage of natural antioxidants obtain by SFE, in food at present a new type of food supplements enriched with the compounds, are marked providing important advantages.

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Chapter 11
Supercritical Fluid Extraction of Carotenoids

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11.1 Introduction

Extraction of valuable bioactive compounds from natural products related to food is an important means of producing value added products, which are considered to have a beneficial effect on health. Natural food additives are gaining more and more importance over synthetic compounds due to their extensive use in food, cosmetics and pharmaceuticals industries. Fruits, vegetables, and sea products contain different kinds of bioactive compounds such as vitamins, carotenoids and polyphenols, which significantly affect their taste, color and nutritive values. The waste and by-products of fruits, vegetables, and sea products are abundant sources of antioxidant carotenoids and therefore the recycling of the byproducts as a source of carotenoids can be of considerable economic benefit to food processors. In addition to their potential health benefit, natural extracts high in antioxidant activity can be added to food products to preserve their color and flavor and hence improve their shelflife.

The extraction and purification processes of these carotenoids are essential as they can be used in the preparation of dietary supplements, nutraceuticals, and functional food ingredients, as well as food additives, pharmaceutical and cosmetic products. Various extraction processes have been in use for the extraction of

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organic compounds from complex solid samples; including Soxhlet extraction, microwave extraction, sonication and pressurized solvent extraction. Conventional separation techniques such as solvent extraction and distillation have the drawback of leaving trace amounts of organic solvents or to cause thermal degradation. They are time consuming and need large amounts of solvents. Supercritical fluid extraction (SFE) using carbon dioxide is a promising alternative for the extraction of value added products since mild temperature used allows the extraction of thermally labile or easily oxidized compounds (Mendiola et al. 2007).

Since the end of the 1970s, supercritical fluids have been used to isolate natural products, but for a long time applications relied on few products only. Now, the development of processes and equipment is beginning to pay off and industries are getting more and more interested in supercritical techniques (Brunner 2005). This interest is also reflected in the high amount of scientific papers dealing with SFE published in recent years. Moreover, industrial applications of SFE have experienced a strong development since the early 1990s in terms of patents (Schutz 2007).

In supercritical fluid, the physicochemical properties of a given fluid, such as density, diffusivity, dielectric constant and viscosity can be easily controlled by changing the pressure or the temperature without ever crossing phase boundaries. Supercritical fluids have liquid like densities and higher diffusion coefficient and low surface tension resulting in an easy penetration of the supercritical solvent into the porous structure of the solid matrix to release the solute.

As will be seen throughout this chapter, the main supercritical solvent used is carbon dioxide. The critical point of carbon dioxide (CO_2) is at 31.06 °C and 7.38 MPa. CO_2 is the solvent of choice for use in SFE because it is, generally regarded as safe (GRAS), non-toxic, non-flammable, inexpensive and its critical temperature and pressure are relatively low which helps in preventing thermal degradation of food components being extracted. The problem with most of the fluids besides CO_2 is that they cannot be obtained in pure form and they are difficult to handle. The advantages of SFE are:

1. Supercritical fluids have a higher diffusion coefficient and lower viscosity than liquids which helps to a more favorable mass transfer;
2. Absence of surface tension allows for their rapid penetration into the pores of solid matrices, which enhances the extraction efficiencies;
3. The extraction conditions may be manipulated by varying temperature and pressure affecting the solubility of the various components in the supercritical fluid;
4. SFE does not leave a chemical residue and is thus an environment friendly separation process;
5. SFE uses carbon dioxide gas, which can be recycled and used again as part of the unit operation.

CO_2 dissolves non-polar or slightly polar compounds and therefore is not a very good solvent for high molecular weight and polar compounds. To increase the solubility of such compounds in supercritical carbon dioxide, small volumes (ranging from 0 to 20 mol%) of modifiers that are generally polar or non-polar

co-solvents (e.g. water, methanol, ethanol, hexane, dichloromethane etc.) may be added thus increasing the solvating power by interacting with the solute. The modifiers can also reduce the analyte-matrix interactions improving their quantitative extraction (Bjorklund and Sparr-Eskilsson 2005).

11.2 Natural Carotenoids, Sources, and Biological Properties

Carotenoids are a group of more than 600 different compounds, with isoprenoid (tetraterpenoid) structure, synthesized by plants, photosynthetic organisms, and some nonphotosynthetic bacteria, yeasts, and molds. Carotenoids exhibit yellow, orange, and red colors but when they are bound to proteins, they acquire green, purple, or blue colors. They are found in a large number of natural products, such as fruits, vegetables, and sea products. Important functions of these compounds include anticancer activity, regulation of the immune system, free radical inactivation and fat peroxidation inhibition. They are categorized into two classes, oxygenated carotenoids called xanthophylls (lutein and zeaxanthin) and unoxygenated carotenoids known as carotenes (α-carotene, β-carotene and lycopene). Common oxygen substituents are the hydroxy (as in β-cryptoxanthin), keto (as in canthaxanthin), epoxy (as in violaxanthin), and aldehyde (as in β-citraurin) groups. Carotenoids can be acyclic (e.g., lycopene), monocyclic (e.g., γ-carotene), or dicyclic (e.g., α- and β-carotene). In nature, carotenoids exist primarily in the more stable all-*trans* (or all E) form, but small amounts of *cis* (or Z) isomers do occur during food processing (Schieber and Carle 2005).

The principal carotenoids found in natural products, together with zeaxanthin, which is not as ubiquitous, is shown in Fig. 11.1. β-Carotene, α-carotene, β-cryptoxanthin, lutein, and lycopene are also the carotenoids most commonly found in human plasma. These carotenoids, together with zeaxanthin, have been shown to have health-promoting effects.

β-Carotene, α-carotene, and β-cryptoxanthin are provitamins A. Structurally, vitamin A (retinol) is essentially one-half of the β-carotene molecule. Consequently, β-carotene is the most potent provitamin A; it is also the most widespread (Rodriguez-Amaya 1989, 1990). Lycopene exhibits the highest antioxidant activity, and its plasma level is slightly higher than that of β-carotene (Di Mascio et al. 1989). The results reported for the antioxidant activity of β-carotene differ widely due to the various test systems and the experimental conditions used (Bohm et al. 2002). The conjugated double-bond systems are responsible for the antioxidant properties of carotenoids, which can act by quenching singlet oxygen formed due to the effect of UV light, scavenging peroxyl radicals, hydrogen transfer, or electron transfer (Stahl and Sies 2002, 2003; Kiokias and Gordon 2004).

The major sources of α-carotene include carrots, tomatoes, and vegetables. β-carotene is present in the same material as α-carotene as well as in paprika and sweet potatoes. Lycopene is found in tomatoes, rosehip, apricots, guavas, water-melon,

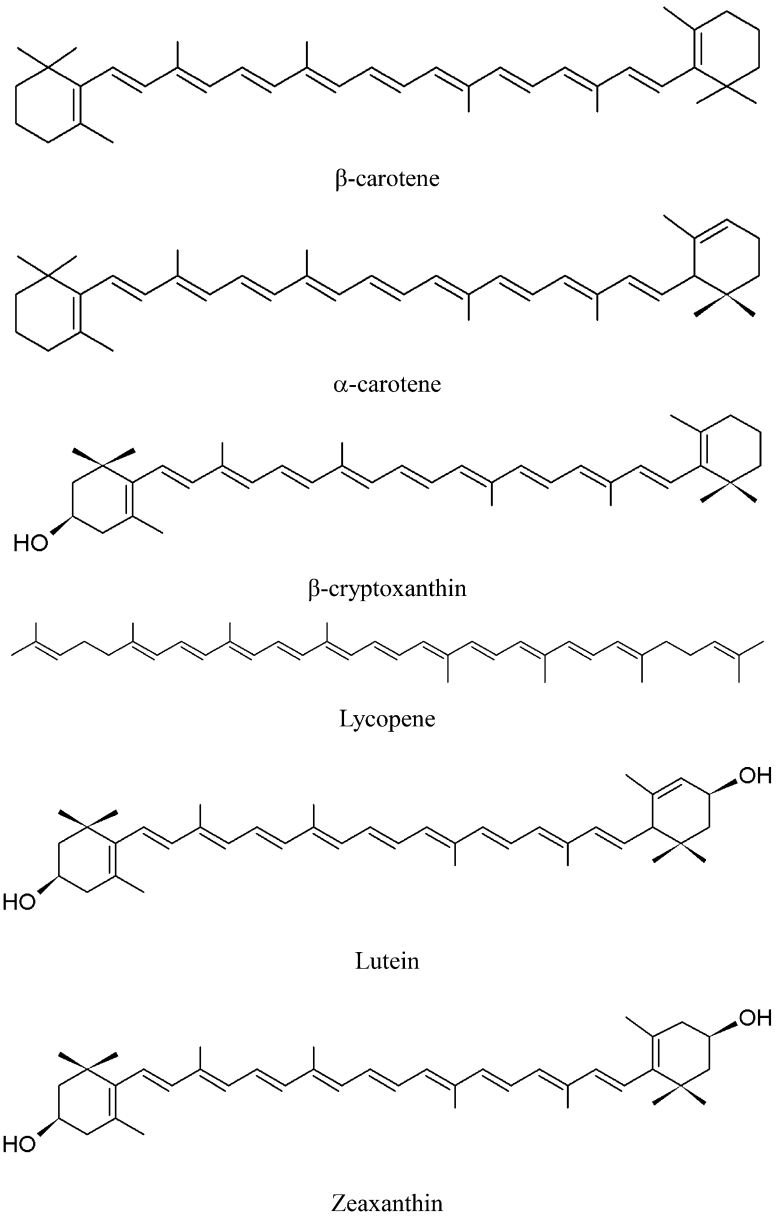


Fig. 11.1 Structures of the principal carotenoids in natural products and zeaxanthin

papayas, and pink grapefruits. β-cryptoxanthin is present in mangos, papaya, peaches, paprika, and oranges, lutein in bananas, egg yolks, spinach, parsley, and marigold flowers; zeaxanthin in paprika; astaxanthin in salmon, the yeast *Phaffia rhodozyma*, and the algae *Haematococcus pluvialis*; and canthaxanthin in carrots. According to

Table 11.1 α-carotene and β-carotene content of various raw materials

Material	Carotenoids			Reference
	α-carotene (μg/g)	β-carotene (μg/g)	Lycopene (μg/g)	
Freeze dried carrots	16.27	33.39	–	Saldana et al. (2006)
Crude carrot oil	137.8–330.3	171.7–386.6	–	Mei and Temelli (2006)
Tomato waste	–	15	31.4	Vagi et al. (2007)
Apricot pomace	–	88	–	Sanal et al. (2004)
Tomato juice	–	0.4	–	Nardo et al. (2009)
Ripe tomato skin	–	–	644	Cadoni et al. (2000)
Rosehip fruit	–	850	17	Machmudah et al. (2008)
Tomato seeds and skin	–	–	7.9	Rozzi et al. (2002)
Dried tomato skin and seeds	–	1,510	820	Machmudah et al. (2012)
Tomato skin	–	–	31	Yi et al. (2009)

Wandawi et al. (1985), carotenoids are mainly found in the fruit skin; the amounts of lycopene and β-carotene are higher in tomato skin and at least five times higher in tomato waste than in other tomato products (Table 11.1). It has been found that apricot pomace, banana peel, and rosehip are also a source of β-carotene. Table 11.1 shows the α-carotene, β-carotene, and lycopene content of various raw materials.

The major biological functions of carotenoids are related to intercellular gap junction communication, cell differentiation, immunoenhancement, and inhibition of muta-genesis. Some carotenoids (α- and β-carotene, β-cryptoxanthin) are precursors of vitamin A and protect against chemical oxidative damage, several kinds of cancer, and age-related macular degeneration. No convincing evidence exists of their protective action against cardiovascular disease (Stahl and Sies 2003; Kiokias and Gordon 2004; Deming et al. 2002; Granado et al. 2003). In vitro studies evidenced that carotenoids can interact with several reactive species and can act as prooxidants, although no documented evidence to date indicates true prooxidant activity in vivo (Lowe et al. 2003). The maximum antioxidant effectiveness of carotenoids in human cells is related to an optimal dose, because higher dose can be less effective or result in cell damage. The relationship between carotenoid intake and cancer has been evaluated, showing an inverse association for lung, colon, breast, and prostate cancer, although negative effects of supplementations have been found (Kiokias and Gordon 2004). Furthermore, it is not clear yet whether the association between diet and disease is due to the specific carotenoid, other micronutrients present in the diet, or to the combined effect of several of these active ingredients. Studies on the mechanism of cancer cell growth inhibition by carotenoids at the protein expression level may involve changes in pathways leading to cell growth or cell death, including hormone and growth factor signaling, regulatory mechanism of cell cycle progression, cell differentiation, and apoptosis (Sharoni et al. 2003).

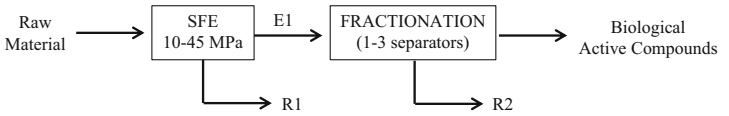
11.3 SFE Processes

Depending on the physical state (solid or liquid) of the phase containing the target compounds, SFE can involve solid-liquid or liquid-liquid mass transfer. solid-liquid extraction is a heterogeneous operation involving the transfer of solutes from the vegetal matrix to a fluid. The extraction rate depends on the external mass transfer, effective solute diffusivity in the solid, solute solubility in the solvent, and solute binding to the solid matrix. Batch extraction and semicontinuous extraction are the most commonly used experimental methods. Extraction by solvent flow through a fixed bed of solid particles allows the recovery of fractions obtained along the extraction period. When a liquid stream has to be processed by SFE, both solubility and interphase mass transfer are relevant. Operation is similar to extraction with conventional solvents, and continuous operation can be carried out in single stage or multistage contact (cross-flow or countercurrent).

Different processing schemes have been proposed for SFE of compounds from natural sources (Diaz-Reinoso et al. 2007). Figure 11.2a–d present simplified flow diagrams of the most usual alternatives, including:

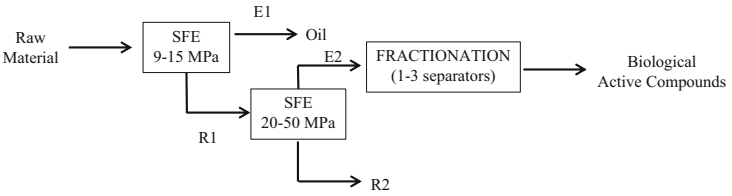
1. Single extraction stage and fractional separation in several separators. The extract obtained in a single extraction step can be fractionated by releasing pressure in the separators. This extraction is widely used for processing solids and for analytical purposes (Dapkevicius et al. 1998; Carvalho et al. 2005; Mezzomo et al. 2013a, b). In this extraction process, modifier may be applied up to 10 % (Mezzomo et al. 2013a, b).
2. Stagewise extraction at progressively increased severity. After a first stage at low severity (<15 MPa, no modifier) to extract nonpolar compounds (essential oil and waxes), further SFE of the solid residue is performed at increased severity (up to 50 MPa, 20 % modifier) to extract more polar compounds (Ashraf-Khorassani and Taylor 2004; Nguyen et al. 1991; Correa et al. 2012). Stepwise extraction needs more solvent than simple extraction with stagewise fractionation of extracts (Mukhopadhyay 2000; Simándi et al. 1998), although the extraction yields can be similar.
3. Combination of conventional solvent and SFE of solid samples. A first SFE stage under low severity conditions can be performed to remove volatile compounds and waxes from the solid substrate (Esquivel et al. 1999; del Valle et al. 2004) before extraction with conventional solvents. For instance, hop oil essences have been extracted by density programmed SFE of hop pellet followed by solid phase extraction using ethanol/water mixtures (Opstaele et al. 2012). A hydrothermal treatment, with environmental and operational advantages derived from the nontoxic character of the solvent, has been used for extracting biologically active compounds from SFE-extracted bamboo (Quitain et al. 2004) and sugars from SFE-extracted pressed palm fiber (Cardenas-Toro et al. 2014).
4. SFE of dry extracts or solid residues. solid-liquid supercritical CO_2 extraction can be employed to purify commercial extracts, dried extracts from conventional

a



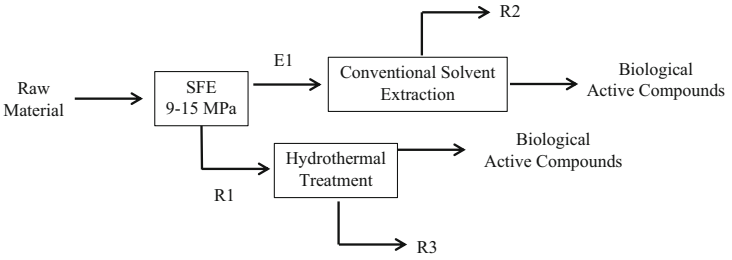
Raw Material	Flowers, Fruits, Leaves, Spices, Medicinal Plants, Seeds, Hulls, Roots	Crustacean, Micro-algae, Tomato, Rosehip	Olive Leaves, Medicinal Plants, Wheat Germ, Seeds
Biological Active Compounds	Phenolics and Terpenoids	Carotenoids	Vitamin E

b



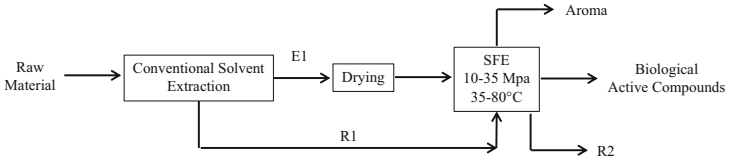
Raw Material	Leaves, Medicinal Plants, Seeds	Paprika, Crustacean
Biological Active Compounds	Phenolics and Terpenoids	Carotenoids

c



Raw Material	Medicinal Plants, Stalks
Biological Active Compounds	Phenolics and Terpenoids

d



Raw Material	Grape Seeds, Grape Pomace
Biological Active Compounds	Phenolics and Terpenoids

Fig. 11.2 Processing schemes for extraction of antioxidant compounds involving supercritical fluid extraction (SFE) stages. E1, E2: extracts; R1, R2, R3: solid residues

solvent extraction, or compounds remaining in the solid residue from conventional solvent extraction. The two first schemes have been proposed for enhancing the antioxidant activity and improving the organoleptic properties (dearomatization) of extracts (López-Sebastián et al. 1998; Hadolin et al. 2004). Improved benefits have been reported for high-molecular-weight compounds, probably due to their concentration and interactions with the matrix (Murga et al. 2000).

Usually, natural raw materials for SFE show both limited contents of the target compounds and low bulk density, making the utilization of large volume extractor necessary (Ribeiro et al. 2001). Because of this, processes involving conventional solvent extraction and further purification of the crude of SFE are comparatively advantageous, as they provide higher yields and/or lower specific CO_2 consumption than direct extraction of the vegetable feedstock.

11.3.1 Effect of Process and Material Parameter on SFE Yield

SFE performance is strongly influenced by prior conditioning of the starting material and by the experimental conditions employed in the extraction and separation. Thus, the nature and properties of vegetable feedstocks or their processing streams (including maturity stage, cultivar, variety, edaphoclimatic conditions) have a substantial effect on the extraction of carotenoids (Guglü-Üstündag and Temelli 2004; Vasapollo et al. 2004) from solid samples. When processing solids, mechanical-thermal conditioning is decisive to facilitate the extraction of intracellular solutes. Reduced particle size favors mass transfer, but too-small particles could limit the performance of fixed beds and grinding may result in losses by volatilization and degradation of active compounds.

Temperature, pressure, solvent flow rate and type of solvent are important process parameters. Origin of the plant material, chemical composition and particle size of the material, different types of tissues (leaves, stems, seeds, etc.) and other variables, like pretreatment and storage conditions, also affect yield and composition of the extract in SFE. Optimization of the process and material parameters are important to provide maximum yields with the highest quality and making the final product suitable for use in foods, cosmetic or pharmaceutical industries.

11.3.1.1 Effect of Temperature

According to Reverchon and De Marco (2006), in supercritical CO_2 extraction, the most important parameter that affects the selectivity and solvent power of the supercritical fluid, which in turn determine the yield of the target compound, is extraction pressure. However, in the supercritical CO_2 extraction of carotenoids

from various fruit matrices, the results of numerous investigations indicate that extraction temperature is the most important parameter (Reverchon and De Marco 2006). The temperatures used in the different studies on the supercritical CO_2 extraction of carotenoids are given in Table 11.2.

Table 11.2 Temperatures and pressures used in various studies on supercritical CO_2 extraction of carotenoids

Sample sources	Temperature (°C)	Pressure (MPa)	Particle size	Reference
Tomato paste waste (dry matter content 24 ± 2 %)	35, 45, 55, 65	20, 25, 30	3 mm	Baysal et al. (2000)
Whole fresh tomatoes, skins and dried seeds of field tomatoes	40, 50, 60, 70, 80	17.24–27.58	Ground and filtered in a vacuum	Cadoni et al. (2000)
Sun dried tomatoes, roasted hazelnuts	60–70	40–45	1 mm, corresponding to 18 mesh	Ciurlia et al. (2009)
Tomato oleoresin obtained from tomato paste by extraction with petroleum ether	40, 50, 60	10–42	N/A (Tomato paste)	De la Fuente et al. (2006)
Tomato skins	40, 50 °C (separation vessel 1), 60 °C (separation vessel 2)	32 MPa, 15 MPa (separation vessel 1), 6 MPa (separation vessel 2)	N/A (Ground)	Del Castillo et al. (2003)
Tomato juice	40, 60, 80	20, 27.5, 35	N/A (Tomato juice)	Egydio et al. (2010)
In natura pitanga fruit	40, 60	10, 15, 20, 25, 30, 35, 40	Mean particle size: 0.376 mm, sieve meshes: 16, 24, 32, 48, 80	Filho et al. (2008)
Lyophilized microalga *Scenedesmus obliquus*	40, 50, 60	15, 20, 25	N/A	Guedes et al. (2013)
Microalgae *Synechococcus* sp.	40, 50, 60	20, 30, 40	N/A	Cardoso et al. (2012)
Corn distiller's dried grains with solubles (DDGS)	50, 60, 70	34.5, 42.1, 49.6	N/A (Ground)	Ciftci et al. (2012)
Tomato skin and pulp without seeds	40	7.7–28.1	N/A (Ground)	Gomez-Prieto et al. (2003)
Tomato industrial waste (mixture and skins and seeds)	40, 60, 80	20, 30	0.15, 0.36, 0.48, 0.70, 0.72 mm	Nobre et al. (2009)

(continued)

Table 11.2 (continued)

Sample sources	Temperature (°C)	Pressure (MPa)	Particle size	Reference
Tomato skins	60, 85, 110	40.53	Tomato skins powdered with sea sand, household blender	Ollanketo et al. (2001)
Tomato seeds and skins (51.6 % dry matter—by-product of steam peeling)	32, 41, 50, 59, 68, 77, 86	13.78–48.26 MPa at 3.45 MPa intervals	N/A	Rozzi et al. (2002)
Tomato waste (skins and seeds—80 % moisture content)	60, 80	25, 30	80 and 345 μm	Sabio et al. (2003)
Tomato peel by-product containing tomato seeds	70–90	20–40	1.05 ± 0.10 mm	Machmudah et al. (2012)
Fresh tomatoes chopped into cubes	40, 70	40	0.5–1 mm	Saldana et al. (2010)
Air-dried tomato skins and seeds (93 % dry matter)	50, 60, 70, 80	25, 30, 35	1 mm, corresponding to 18 mesh	Shi et al. (2009a)
Tomato skins	45, 60, 75	20, 25, 30, 35, 40	1 mm, corresponding to 18 mesh	Shi et al. (2009b)
Dried tomato skins	70, 80, 90, 100	20, 30, 40, 50	Ground prior to extraction	Topal et al. (2006)
Dried tomato pomace containing skins and seeds of ripe tomato	40, 60, 80	30, 38, 46	0.3, 0.4, 0.6 mm	Vagi et al. (2007)
Sun dried tomatoes (50 % moisture)	45–70	33.5–45	1 mm, corresponding to 18 mesh	Reverchon and De Marco (2006)
Pink shrimp residue	40, 60	10, 20, 30	N/A (Ground)	Mezzomo et al. (2013b)
Freeze-dried watermelon cubes ground into a powder	60, 70, 80, 90	20.7, 27.6, 34.5, 41.4	Ground with a mortar and pestle to a coarse powder	Vaughn et al. (2008)
Ground-dried *Haematococcus pluvialis*	40–80	20–55	N/A (Ground)	Machmudah et al. (2006)
Air-dried tomato skins and seeds (93 % dry matter)	40, 50, 60, 70, 80, 90, 100	20, 25, 30, 35, 40	1 mm, corresponding to 18 mesh	Yi et al. (2009)

The effect of temperature confounds as there is a competing solubility effect caused by the increase in vapor pressure and the decrease in density upon the increase in temperature. The density of CO_2 at constant pressure is decreased with increasing temperature and leads to reduction of fluid solvent power. In a work done by Mezzomo et al. (2010), it was observed that at 10 MPa, raising extraction temperature decreased the yield of peach almond oil, due to the reduction in solvent density while at higher pressures (above 200 bar), the increase in the extracting temperature provided an increase in the yield, despite the reduction in solvent density. These opposite effects on the overall extraction yields were responsible for the inversion of the yield isotherms. Therefore, considering the crossover characteristic, Mezzomo et al. (2010) suggested that, at pressures below the crossover pressure, the density effect was dominant, while at higher pressures the solute vapor pressure was the leading mechanism affecting the extraction process.

With the increase in temperature, there is an improvement in the mass transfer and in the extraction yield. The increase in temperature causes enhancement of the vapor pressure of the extractable compounds which is more significant than the reduction in the solvent density, increasing consequently the overall extraction yield. Thus, the tendency of the compounds to be extracted is increased as they can pass to the supercritical fluid phase. This phenomenon generally can be observed in the extraction of carotenoids. Figure 11.3 shows the effect of extraction temperature on the β-carotene and lycopene recovery extracted from tomato peel by-product. β-carotene recovery decreased with increasing temperature, while lycopene recovery increased with the increase in temperature.

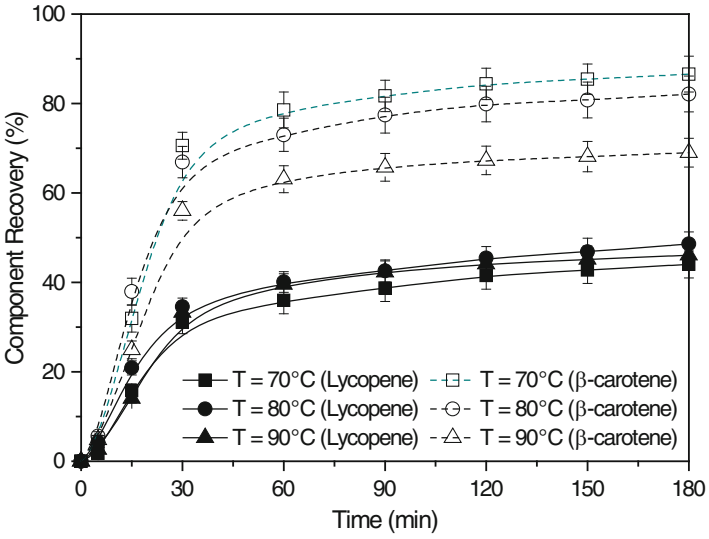


Fig. 11.3 Effect of temperature on the recovery of lycopene and β-carotene at 40 MPa, particle size of (1.05 ± 0.10) mm and 3 mL/min (Machmudah et al. 2012)

Most of the carotenoids occur naturally as *trans*-isomer in plants. However, during processing of food, *cis*-isomers may increase due to the isomerization of the *trans*-isomer of carotenoids. Factors involving heat, light, and structural differences affect the isomerization of carotenoids in foods. All-*trans*-β-carotene is very unstable and can be easily isomerized into *cis*-isomers, when exposed to heat and light. Inversely, lycopene commonly occurs in the all-trans configuration, which is the most thermodynamically stable form Gomez-Prieto et al. (2003; Nobre et al. 2009). Mezzomo et al. (2010) have stated that due to the thermodynamic stability of *trans*-lycopene, there is clearly an interest in avoiding the isomerization of *trans*-lycopene into its *cis*-form when lycopene is obtained for subsequent incorporation into functional foods and nutraceuticals. The thermodynamic stability of *trans*-lycopene also makes it easier to manipulate and incorporate it into functional foods and nutraceuticals, when compared to *cis*-lycopene (Nobre et al. 2009).

According to Konar et al. (2012), in SFE, extraction under low pressure and temperature conditions can inhibit *trans-cis* isomerization but the products should be protected against heat and oxygen. Solubility increase depends on both pressure and temperature and it has a significant effect on extraction efficiency. In the extraction of lycopene, (60–70) °C was reported to be the optimum temperature while significant thermal degradation was observed at 80 °C. The solubility of lycopene in $SCCO_2$ is at higher levels at low temperatures. However, extraction of lycopene from tomatoes by superctritical CO_2 results in less isomerization and decomposition. Increasing temperatures increases significantly degradation, which occurs mainly through oxidation without isomerization in the range (25–50) °C and with isomerization at temperatures in the range (75–150) °C.

In a study conducted by Nobre et al. (2012), it was demonstrated that there was a slight increase in the recovery of the carotenoid when the temperature increased from (40 to 60) °C and remained almost the same with the further rise of the temperature to 80 °C. That increase is possibly due to some isomerization taking place at the higher temperature.

Similarly, Shi et al. (2009a) observed that at extraction temperatures of 80 °C and higher, there was a decline in the solubility curve of lycopene in supercritical CO_2, and attributed this to thermally-induced lycopene degradation. Yi et al. (2009) stated that although an elevation of extraction temperature would increase lycopene yields, the instability of lycopene at high temperatures would cause the compound to undergo degradation and isomerization. In their study on the extraction of lycopene from air-dried tomato skins and seeds, they found that there was no significant change in the ratio of *trans*-lycopene to *cis*-lycopene when supercritical CO_2 extraction was carried out at temperatures below 70 °C. When the extraction temperature was increased above 70 °C, then the composition of lycopene isomers in the extract changed. When the extraction temperature was raised from (50 to 90) °C, the ratio of *trans*-lycopene to *cis*-lycopene changed from 1.67 to 1.40 (Yi et al. 2009). They also found a higher increase of *cis*-lycopene compared to *trans*-lycopene accompanying the rise in extraction temperature. When the extraction temperature was increased to 90 °C, total lycopene content of the extract was 1.18 times higher. *Trans*-lycopene content in the extract increased only 1.03 times, while that of *cis*-lycopene—2.13 times. They concluded that the higher increase of

cis-lycopene compared to that of *trans*-lycopene and to the total lycopene content was a result of *trans*-lycopene isomerization into the *cis*-isomer form (Shi et al. 2009a). Our group also reported that lycopene recovery was decreased when the extraction temperature was increased beyond 80 °C.

Baysal et al. (2000) have stated that 100 % recovery of carotenes extracted by supercritical CO_2 is not possible due to the degradation that the carotenes undergo during the extraction process. Ollanketo et al. (2001), however, reported a full 100 % recovery of lycopene extracted from tomato skins by supercritical CO_2 which was achieved at an extraction temperature of 110 °C and after an extraction time of 50 min. This study however, remains the only one in the literature so far that has reports 100 % recovery of lycopene by supercritical CO_2 extraction. Furthermore, extraction at such high temperatures may result in destruction of the raw sample. Yi et al. (2009) reported that the raw material utilized in their study became scorched after 90 min of extraction at 100 °C.

As reported by Gomez-Prieto et al. (2003) even though extraction temperature was maintained as low as 40 °C, the presence of *cis*-lycopene could still be observed; the authors attributed this outcome to the ease with which lycopene undergoes *trans*- to *cis*-isomerization when exposed to light and oxygen.

The ease with which lycopene undergoes isomerization and degradation when exposed to light and oxygen means that steps must be taken at all stages of the experiments involving supercritical CO_2 extraction. According to Cadoni et al. (2000), the extraction methods reported in the literature are difficult, poorly reproducible and subject to errors on account of loss of lycopene during extraction via degradation and isomerization. In their experimental methods, Shi et al. (2009a) minimized exposure of the raw material and extracts to light, heat and air. However, it was improbable that all of the thermal and photochemical degradation could be avoided, thus adding uncertainty to the data gathered. A common method to avoid isomerization and degradation by photo-oxidation is by performing the extraction and analysis under dim light (Yi et al. 2009; Ciurlia et al. 2009; Shi et al. 2009b). Shi et al. (2009a) also collected the samples obtained by supercritical CO_2 extraction in 25 mL brown vials to prevent UV-activated degradation of the samples. Storage of the raw materials prepared prior to supercritical CO_2 extraction, as well as of subsequent extracted compounds, at sub-zero temperatures is another common measure taken to prevent isomerization and degradation of lycopene, with −20 °C being the most common temperature utilized for this purpose (Rozzi et al. 2002; Machmudah et al. 2012; Gomez-Prieto et al. 2003; Nobre et al. 2009; Yi et al. 2009; De la Fuente et al. 2006; Del Castillo et al. 2003; Sabio et al. 2003; Saldana et al. 2010; Vaughn et al. 2008).

Another obvious measure that can be taken to minimize isomerization and degradation of carotenoids during extraction by supercritical CO_2 is to modify the extraction temperature itself. The extraction temperature for thermolabile compounds has to be fixed between (35 and 60) °C, which is in the vicinity of the critical point of CO_2 and still as low as possible to avoid degradation of the compound (Reverchon and De Marco 2006). To minimize isomerization of lycopene, Saldana et al. (2010) utilized temperatures of (40 and 70) °C in their study, while Cadoni et al. (2000) performed initial extractions of lycopene with supercritical CO_2 at 40 °C to avoid degradation of

the compound. Taking this into account it can be concluded that SFE under mild working conditions can avoid *trans*- to *cis*- isomerization.

11.3.1.2 Effect of Pressure

The effects of pressure on supercritical CO_2 extraction of carotenoids from plant matrices seem to be similar to those of temperature. The various pressures used in studies on the supercritical CO_2 extraction of carotenoids are given in Table 11.2.

Increases in pressure at constant temperature and in the density of the solvent alter the solute solubility and the vapor pressure of the solute decreases. At elevated pressure, the magnitude of such density change becomes smaller and the solute vapor pressure change becomes more effective; the latter can easily overcome the effect of solvent density change on the extraction rate. A higher recovery of volatile fractions and a lower recovery of non-volatile fractions are obtained with increasing pressure in the process. Therefore, the composition of the extract can be controlled by regulating pressure. At high pressure, there is an increase in the extraction yield with temperature rise which is due to the enhancement in solute vapor pressure and the reduction of solvent density. Similar results have been obtained in studies for astaxanthin and lycopene extraction conducted by our group (Machmudah et al. 2006, 2008, 2012). However, at low pressures, the decrease in solvent power due to reduction in solvent density is the leading mechanisms. These opposite effects cause the inversion of the extraction yield isotherms, a phenomenon known as crossover yield isotherms or retrograde phenomenon.

The majority of the literatures indicate that an increase in the extraction pressure of the supercritical CO_2 leads to an increase in the amount of carotenoids extracted (Vagi et al. 2007; Machmudah et al. 2006, 2008, 2012; Murga et al. 2000; Vasapollo et al. 2004; Reverchon and De Marco 2006; Gomez-Prieto et al. 2003; Yi et al. 2009; Baysal et al. 2000; Shi et al. 2009b; Sabio et al. 2003). However, some studies, while confirming that carotenoids recoveries were higher when extraction pressures were higher, also state that the differences in the amount of carotenoids recovered were not statistically significant. Baysal et al. (2000) utilized pressures of (20–30) MPa in the extraction of lycopene from tomato paste waste and found no significant difference when the pressure was changed in that range. They found that the highest yields of carotenoids were extracted at 30 MPa and suggested that carotenoids recoveries could be improved by extractions of up to 40 MPa. Similarly, Egydio et al. (2010) found that while pressure did not have a statistically significant effect on the lycopene extraction yield, the highest lycopene yield was obtained at the highest pressure of 35 MPa used in the study. Shi et al. (2009b) also found that the highest pressure utilized in their study, 35 MPa, led to the highest yield of lycopene. In the case of astaxanthin extraction from micro-algae, pressure did not significantly affect the astaxanthin extraction yield in the pressure range of (30–50) MPa (Machmudah et al. 2006). The astaxanthin extraction yield dramatically increased at pressure of 55 MPa as shown in Fig. 11.4.

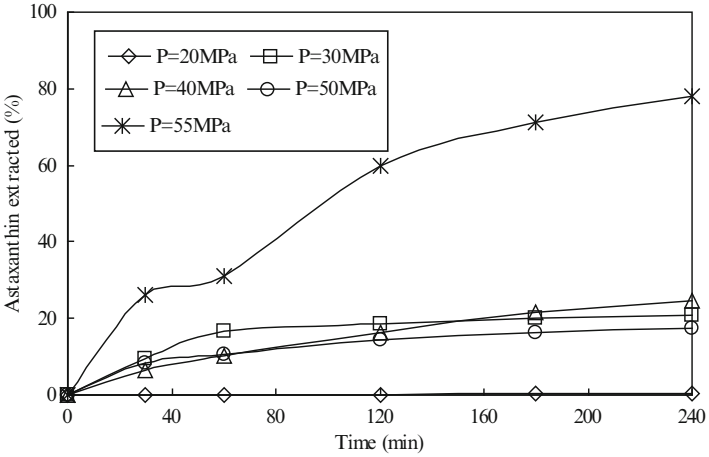


Fig. 11.4 Effect of pressure on astaxanthin extracted as function of time at 343 K and 3 mL/min (Machmudah et al. 2006)

The increase in the recovery of carotenoids at higher extraction pressures is mainly due to the increase in the density of supercritical CO_2 when pressure is increased (Vagi et al. 2007; Machmudah et al. 2006, 2008, 2012; Murga et al. 2000; Vasapollo et al. 2004; Reverchon and De Marco 2006; Gomez-Prieto et al. 2003; Yi et al. 2009; Baysal et al. 2000; Shi et al. 2009b; Sabio et al. 2003). At higher densities, the dissolution of a solute into supercritical CO_2 is enhanced due to the greater interaction between the solute and the supercritical fluid (Machmudah et al. 2006, 2008, 2012; Shi et al. 2009a). According to Reverchon and De Marco (2006), the solvent power for supercritical CO_2 is often expressed in terms of its density at given operating conditions.

Some studies demonstrated that while pressure increase leads to an increase in the recovery of carotenoids, yet, beyond an optimum point, a further increase in pressure leads to a decrease in the yield of lycopene. For example, Machmudah et al. (2012), found that when the extraction temperature and pressure were elevated to 90 °C and 30 MPa, the lycopene recovery was 51 %, which was the maximum recovery achieved. Further increases in pressure beyond this point led to a decrease in lycopene recovery. Similarly, Topal et al. (2006) found that increasing pressure from (20 to 40) MPa resulted in a gradual increase in lycopene recovery, but a further increase in pressure from (40 to 50) MPa did not improve the recovery of lycopene. Since the optimum was not at the maximum pressure utilized in the study, Rozzi et al. (2002) attributed the decrease in carotenoids yield to the decrease in the ability of supercritical CO_2 to diffuse through the sample. This decrease in the diffusivity of the supercritical fluid is due to the increase in its density with an increase in pressure. Machmudah et al. (2012) observed that sample residue, obtained in the extraction column after supercritical CO_2 extraction had been carried out, was compacted. This led them to attribute the decrease in lycopene recovery to compaction of the sample at higher pressure, which leads to channeling of the supercritical CO_2 and consequently inhibits its diffusion into the sample.

In a study on the supercritical CO_2 extraction of lycopene from watermelon, Vaughn et al. (2008) found that pressure had a negligible effect on lycopene yields, while solvent density had a weak effect. The results of this study seem to run against the general trend observed in the literature on the supercritical CO_2 extraction of lycopene from plant matrices.

11.3.1.3 Effect of Co-solvent Modifier

Supercritical CO_2 has good solvent properties and is often used for extraction of non-polar compounds and some polar compounds. Sometimes the extraction must be carried out without modifier as in the case of extraction of vitamin E and provitamin A being lipophillic compounds, supercritical CO_2 is considered a relatively good solvent for extraction. Pure CO_2 is not widely used for the extraction of hydrophilic compounds and a common practice in SFE is to change the polarity of the supercritical fluid by addition of small amounts of organic co-solvents and thereby increasing the solvating power towards the target compounds. The co-solvents interact strongly with analytes (hydrogen-bonding, dipole-dipole and others polarity interactions) which results in significant improvement in the extraction yields. Most commonly used co-solvents in SFE are ethanol and methanol. There are two main procedures by which co-solvents or modifiers can be incorporated in the SFE process: by either mixing of the modifier with the CO_2 flow or by mixing the modifier with the raw material in the extraction cell. In a work done in our group (Ruen-ngam et al. 2012), the use of ethanol as co-solvent with CO_2 increased the extraction yield of astaxanthin extract by rupturing the solute/solid matrix interactions, and substituting co-solvent molecules in the solid active sites. Machmudah et al. (2006) also showed that ethanol concentration increased the astaxanthin content of the extracts from micro-algae due to increased carotenoid-alcohol interactions which increase the solvating power of CO_2. In addition, ethanol increases the bulk density of supercritical CO_2 (due to the higher density of the co-solvent) and the clustering of supercritical CO_2 molecules around the co-solvent. Figure 11.5 shows the result, where the optimum concentration is 5 %. However, in a work done by Lamin et al. (2008), recovery of lycopene increased in the higher temperature regions where the ethanol concentration was low but higher temperature had a detrimental effect on lycopene as a result of instantaneous heating. Dependence of solubility enhancement on temperature was reported by Vega et al. (1996), who also illustrated higher ethanol concentration to be less effective as temperature increases. This phenomenon was also proven by Baysal et al. (2000) with a higher recovery at 5 % modifier concentration compared to 10 % at a temperature of 40 °C. In contrast, Nobre et al. (2013) found that the highest recovery of pigment compounds (carotenoids) extracted from *Nannochloropsis* sp. microalga was obtained at 20 % (w/w) ethanol modifier.

Non-polar compounds such as hydrocarbons are most efficiently dissolved in relatively non-polar solvents while polar compounds are most efficiently dissolved in relatively polar solvents (Ollanketo et al. 2001). Being a non-polar compound,

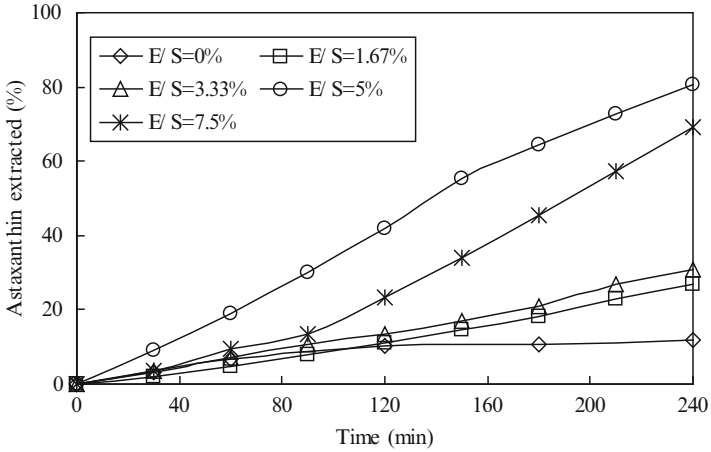


Fig. 11.5 Effect of entrainer concentration on astaxanthin extracted as a function of time at 40 MPa and 343 K (Machmudah et al. 2006)

carotenoids are insoluble in water but soluble in organic solvents such as acetone, benzene, chloroform, ethanol, n-hexane, methanol, methylene chloride and tetrahydrofuran (THF) (Vasapollo et al. 2004; Gomez-Prieto et al. 2003; Nobre et al. 2009; Baysal et al. 2000; Cadoni et al. 2000; Ciurlia et al. 2009; Topal et al. 2006). Extraction of carotenoids from plant sources, including β-carotene and lycopene, is usually carried out by chemical extraction utilizing organic solvents (Reverchon and De Marco 2006; Nobre et al. 2009; Ciurlia et al. 2009). However, the utilization of organic solvents to extract carotenoids poses a multitude of problems. Organic solvents tend to be toxic, hazardous to handle, difficult to dispose of, and require long extraction periods (Machmudah et al. 2012; Vasapollo et al. 2004; Nobre et al. 2009; Ollanketo et al. 2001; Cadoni et al. 2000; Ciurlia et al. 2009; Sabio et al. 2003; Saldana et al. 2010; Topal et al. 2006). They also tend to be expensive, and the amount of organic solvent required to extract the carotenoids will have a direct influence on the total cost of the final carotenoids extract (Vasapollo et al. 2004; Baysal et al. 2000; Sabio et al. 2003). The usage of organic solvents to extract carotenoids also requires that the solvents must then be removed from the extract via heat treatment, which in turn leads to residual presence of traces of the organic solvent in the carotenoids extract (Vagi et al. 2007; Machmudah et al. 2012; Vasapollo et al. 2004; Nobre et al. 2009; Ollanketo et al. 2001; Cadoni et al. 2000; Saldana et al. 2010; Topal et al. 2006). If the organic solvent used to extract the carotenoids is toxic, then the presence of residual traces in the extract will render it unsuitable for human consumption. Due to the non-toxic properties of water and ethanol, these two compounds may be used instead of organic solvents for the extraction of carotenoids (Shi et al. 2009b). The drawback, however, lies in the polar nature of the two compounds, which makes them less-than-suitable for the extraction of carotenoids.

With an increase in concern over the safety of food and pharmaceutical products, the non-toxic nature of supercritical CO_2, as well as the capability of the process to

produce solvent-free extracts, makes it a particularly suitable green alternative method for carotenoids extraction (Vagi et al. 2007; Machmudah et al. 2012; Vasapollo et al. 2004; Nobre et al. 2009; Ollanketo et al. 2001; Saldana et al. 2010).

Carotenoids display low to moderate solubility in supercritical CO_2, depending on their molecular weight and number of polar bonds (Reverchon and De Marco 2006). To increase the solvent power of supercritical CO_2, the use of co-solvents or modifiers has been applied to the process. Co-solvents are added to the supercritical CO_2 stream as it flows into the extraction chamber/cell, while modifiers are added directly to the sample or mixed in with the sample before extraction occurs (Rozzi et al. 2002).

Cadoni et al. (2000) tested the effects on the extraction of carotenoids from fresh and dried tomatoes of several organic solvents (hexane, ethyl ether, ethanol and chloroform) as modifiers. The authors found that chloroform modifier produced the best yield. Ollanketo et al. (2001) also tested the effects of modifiers on lycopene extraction from dried tomatoes, namely acetone, methanol, ethanol, hexane, dichloromethane and water. They found that the best lycopene recovery was obtained with using acetone as modifier, with 100 % recovery in 50 min. Acetone also accelerated the recovery, with 94 % of lycopene obtained in just 15 min.

Vasapollo et al. (2004) tested the effects of different vegetable oils utilized as modifiers in the supercritical extraction of lycopene from dried tomatoes. Although in the study the vegetable oils were called “co-solvents”, they were in fact modifiers, based on the definition given by Rozzi et al. (2002), as the vegetable oil was manually inserted into the extractor with the tomato powder sample. The vegetable oils tested were almond, peanut, hazelnut and sunflower seed oil, with only hazelnut oil giving higher lycopene recovery. The amount of lycopene extracted also increased when the amount of hazelnut oil was increased from (1 to 20) % (w/w). The authors found that higher amounts of oil resulted in a more dilute lycopene extract and finally settling on a modifier amount of 10 % (w/w), compared the results of lycopene yield from extraction with the presence of a modifier to that without a modifier. The highest lycopene yield of 60 % was obtained in the presence of a modifier and the presence of hazelnut oil also helped to keep the lycopene stable and inhibited degradation, in addition to increasing solubility by enhancing the transport of lycopene molecules from the solid phase into the supercritical carbon dioxide phase (Vasapollo et al. 2004; Ciurlia et al. 2009).

Machmudah et al. (2012) added tomato seeds as a source of seed oil; the latter acted as a modifier of carotenoids extraction from tomato peel by-product. They found that the increasing seeds to peel ratio from 50 to 63 enhanced carotenoids recovery. However, the increasing seeds to peel ratio from 63 to 70 decreased the carotenoids recovery. They explained that higher concentration of extracted seed oil might hinder supercritical CO_2 transport penetration into the solid matrixes. Moreover, the effect of vegetable oil as a modifier is limited due to its low solubility in CO_2 (Sovova et al. 2001). Furthermore, the recovery was significantly decreased.

One of the effects that co-solvent addition may have upon the sample matrix is swelling, which in turn may affect the cellular structure of the sample allowing a better access of the solvent to the carotenoids molecules, and thus improving the

diffusion of carotenoids into the supercritical CO_2 (Ciurlia et al. 2009; Saldana et al. 2010). The use of ethanol as a co-solvent improves extraction by diluting the lycopene extract, reducing its viscosity and facilitating its flow through the extractor (Ciurlia et al. 2009). The use of water as a co-solvent, meanwhile, improves lycopene extraction by swelling the sample matrix. Olive oil, when used as a co-solvent, also improves lycopene extraction by swelling the matrix and increasing the mass transfer rate (Ciurlia et al. 2009). Saldana et al. (2010) used canola oil as a co-solvent; and they reported that the driving force is increased, as evinced by the reduced chemical potential, due to molecular interactions between the triglycerides of canola oil and lycopene. When olive oil and ethanol are combined as modifiers, the increase in lycopene extraction may be caused by ethanol adsorption on the polar sites in the sample matrix and by lycopene increased solubility in the olive oil (Ciurlia et al. 2009). Thus, two effects synergize to increase lycopene recovery. Also, the olive oil molecules may build hydrogen bonds with ethanol molecules, resulting in a greater interaction between the supercritical CO_2 and the co-solvent mixture with the sample matrix (Ciurlia et al. 2009).

While the general trend reported in the literature is that co-solvents and modifiers have a positive effect on lycopene yield, Reverchon and De Marco (2006) reported negligible influence of co-solvents on the recovery of carotenoids. Furthermore, problems connected to solvent elimination are reintroduced with the use of co-solvents and modifiers, since they are extracted along with the compound of interest. When chloroform was used as a modifier to extract carotenoids, traces of chloroform were found in the extract upon conducting GCMS analysis (Cadoni et al. 2000). This prompted the authors to attempt supercritical CO_2 extraction of carotenoids without the use of any modifiers.

There have been a number of subsequent studies conducted where the objective was to extract carotenoids using supercritical CO_2 without the use of any modifier or co-solvent (Rozzi et al. 2002; Machmudah et al. 2012; Gomez-Prieto et al. 2003; Topal et al. 2006). Reverchon and De Marco (2006) stated that applying higher pressures to supercritical CO_2 extraction of carotenoids can compensate the absence of co-solvent. Rozzi et al. (2002) and Topal et al. (2006), based on the results of their respective studies, showed that it is possible to extract carotenoids with supercritical CO_2 at optimum conditions without the use of co-solvents or modifiers.

However, when edible vegetable oils, such as canola and olive oil, are used as co-solvents the carotenoids will be solubilized in the vegetable oil which will be extracted as well. Such carotenoids enriched-edible oil products can be used in a variety of food and nutraceutical products (Shi et al. 2009b; Saldana et al. 2010).

11.3.1.4 Effect of Extraction Time

Extraction time in SFE is one of the most important variables as the efficiency of the recovery yield can be enhanced if the contact of the supercritical solvent with the feed material is maximized. Analysis of the process is performed considering

the overall extraction curve (yield vs extraction time) which gives information regarding time required to realize an economical and advantageous extraction process. The curve presents three stages: a constant extraction rate period, where the solute is easily transferred from a solid to fluid phase; a falling extraction rate period, and finally, the diffusion controlled rate period. Minimum cost of manufacturing is obtained in extraction times close to the end of the constant extraction rate region, in which shortest time and highest rates of recovery of target compounds are observed (Rosa and Meireles 2005). Extraction time also depends upon the type of material, solute concentrations in the matrix, CO_2 flow rate and operation conditions. In a work done by Sanal et al. (2004), longer extraction time resulted in lesser recovery of β-carotene, possibly due to degradation.

Extraction time, which is the total time the supercritical CO_2 extraction is carried out, is also an important parameter that needs to be optimized for ensuring a complete supercritical CO_2 extraction of carotenoids and hence maximizing carotenoids yields (Vasapollo et al. 2004; Reverchon and De Marco 2006; Machmudah et al. 2006; Topal et al. 2006). However, if the amount of supercritical CO_2available for a particular extraction is limited, then the completeness of the extraction process is determined by that amount. In such cases, a high flow rate will result in a shorter extraction time, and *vice versa*. However, if the amount of carbon dioxide available for a supercritical extraction process is not a limiting factor, then the length of the extraction time may be set according to the discretion of the researchers carrying out the extraction. While increasing the extraction time generally leads to more complete extractions of carotenoids, long extraction periods may produce the adverse effect of carotenoids degradation and isomerization due to temperature effects (Gomez-Prieto et al. 2003; Nobre et al. 2009). Baysal et al. (2000) examined the effect of extraction time at (60, 120 and 180) min and found that the highest carotenoids yield was obtained at 120 min. According to them, it was possible that 60 min of extraction time was insufficient for a complete extraction, while thermal degradation occurring at 180 min of extraction led to lowered yields of carotenoids. Reducing the extraction time could also reduce costs as well as improve energy efficiency (Baysal et al. 2000; Ciurlia et al. 2009). A similar result was reported by Kha et al. (2014). They found that carotenoids content in the extract rapidly increased during extraction time of (60–120) min, but slowly increased beyond 120 min because most of the carotenoids had been already extracted.

11.3.1.5 Effect of Flow Rate

The mass transfer process is controlled by the equilibrium between the solid and the fluid phase. At the beginning of the extraction, when the flow rate is increased, the recovery of the extract is faster; at the end of extraction at the lowest flow-rates the recovery is almost the same. Upon increasing the flow rate, the thickness of the film layer around the solid particles is reduced; mass transfer resistance surrounding the particle thus becomes small which leads to an increase in extraction yield with increase of the flow rate (Doker et al. 2004). A similar effect was observed by Machmudah et al. (2006) in their studies; the authors demonstrated that CO_2 flow

rate had a positive and significant effect on the extracted astaxanthin yield. The fact that the highest flow-rate results in the lowest recovery could be due to channeling effects and the impossibility of reaching equilibrium at such a high flow-rate (Rozzi et al. 2002; Nobre et al. 2009; Saldana et al. 2010). This calls for performing an optimization with the view to find the optimum value for the flow rate as a compromise to the amount of CO_2 used. The flow rates and extraction times used in various studies on the supercritical CO_2 extraction of carotenoids are given in Table 11.3.

Bearing a similarity to temperature and pressure, the effect of increasing the flow rate of supercritical CO_2 leads to higher carotenoids yields. A further increase of the flow rate beyond an optimal point, however, leads to lower carotenoids yields. Topal et al. (2006) tested supercritical CO_2 flow rates ranging from (1.5 to 4.5) mL/min and found the highest lycopene yield at a flow rate of 2.5 mL/min. When the flow rate was increased further from (2.5 to 4.5) mL/min, it resulted in a decrease in the amount lycopene extracted. Rozzi et al. (2002) examined the effect of supercritical CO_2 flow rate on carotenoids extraction from tomato seeds and skins at values ranging from (2.5 to 15) mL/min. Experiments where flow rate effects were tested were performed by holding the temperature, pressure and CO_2 volume constant while increasing the supercritical CO_2 flow rate used for each extraction. It was found that as the flow rate was increased from (2.5 to 15) mL/min, the yield of lycopene decreased, with the highest lycopene yield of 61 % obtained at 2.5 mL/min and recoveries of less than 8 % when flow rates were higher than 10 mL/min. Since flow rate values of less than 2.5 mL/min were not examined in the study, it is uncertain whether that was the optimum value or, alternatively lower flow rates might yield higher amounts of lycopene.

Nobre et al. (2009) studied the effect of supercritical CO_2 flow rate on the extraction of *trans*-lycopene from Portuguese tomato industrial waste at three different values of (0.26, 0.59 and 1.05) g/min. The highest amount of *trans*-lycopene, 93 %, was extracted at a flow rate value of 0.59 g/min. The authors also verified that increasing the flow rate from (0.59 to 1.05) g/min leads to a decrease in the recovery of *trans*-lycopene. Sabio et al. (2003) tested the effects of two supercritical CO_2 flow rates, namely (13.2 and 22.5) g/min, on the extraction of carotenoids from tomato processing waste and found that the higher yield of carotenoids was obtained at the lower flow rate.

However, Yi et al. (2009) found that while increasing the supercritical CO_2 flow rate from (1.0 to 2.0) mL/min resulted in an increase in the amount of lycopene extracted from tomato skins, such an increase was not significant ($P > 0.05$). Baysal et al. (Yi et al. 2009), meanwhile, tested three flow rates of (33.33, 66.67 and 133.33) g/min, respectively, on the supercritical CO_2 extraction of lycopene from tomato paste waste and found optimum extraction yields at 66.67 g/min. The yield of lycopene obtained at a flow rate of 133.33 g/min however, was found not to be significantly different from the yield obtained at 66.67 g/min. Moreover, for the astaxanthin extraction from micro-algae, an increase in the flow rates from (2 to 4) mL/min resulted in an extraction yield increase (Machmudah et al. 2006).

The decrease in the extraction of lycopene yields with an increase in flow rate of supercritical CO_2 can be attributed to channeling effects (Rozzi et al. 2002; Nobre et al. 2009; Saldana et al. 2010). When the supercritical CO_2 flow rate is increased,

it flows through the sample at high velocities and instead of diffusing through the sample matrix, it flows around the sample through channels, thus limiting the contact necessary for extraction of carotenoids.

The effect of flow rate on the supercritical CO_2 extraction of lycopene is closely intertwined to residence time. The residence time is the length of period the supercritical CO_2 remains in the extraction chamber when flowing through it. The smaller the value of the flow rate and the larger the volume of the extraction chamber, the longer the residence time. The higher the flow rate and the smaller the volume of the extraction chamber, the shorter is the residence time. A higher residence time allows for the supercritical CO_2 to remain in the extraction chamber for longer periods, thus allowing it to remain in contact with and diffuse through the pores of the sample matrix, resulting in higher extractions of carotenoids (Rozzi et al. 2002; Baysal et al. 2000; Saldana et al. 2010). Since the amount of CO_2 used was constant for each flow rate value, it was hypothesized that the residence time and the intensity of mixing of the supercritical CO_2 with the sample matrix were the only variables, while the separation characteristics remained constant. While lowering the supercritical CO_2 flow rate may increase the residence time, flow rates which are too low will result in insufficient amounts of supercritical CO_2 required to extract the carotenoids, leading to lower carotenoids yields, as discovered by Topal et al. (2006) with a flow rate of 1.5 mL/min.

11.3.1.6 Effect of Particle Size

Extraction yield increases with decreasing particle size, as grinding before extraction not only increases the interfacial area but also releases solutes by destroying the particles inner structures, which results in higher extraction rate. The intraparticle diffusion resistance is smaller for smaller particle size due to the shorter diffusion path. An increasing amount of the extract versus particle size was noticed and it was concluded that cellular structures should be broken to get a complete extraction of substances [???]. An example of the effect of particle size on carotenoids recovery is shown in Fig. 11.6. As seen, the recovery of both lycopene and β-carotene significantly increases with decreasing particle size. Decreasing the particle size decreases mass transfer resistance and consequently increases the recovery of lycopene and β-carotene. The various particle sizes used in studies on the supercritical CO_2 extraction of carotenoids are given in Table 11.2.

11.3.1.7 Effect of Moisture Content

The moisture content of the feed plays an important role during the extraction of carotenoids. The extraction of carotenoids by supercritical CO_2 appears to be related to the amount of moisture present within the sample and how it interacts with the sample matrix and the supercritical CO_2.

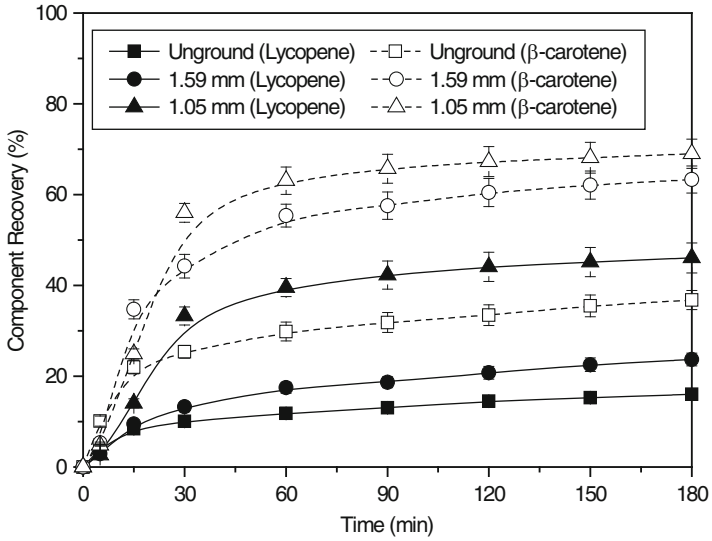


Fig. 11.6 Effect of particle size on the recovery of lycopene and β-carotene (Machmudah et al. 2012)

Table 11.3 Flow rates, extraction times and mass of raw material used in various studies on supercritical CO_2 of carotenoids

Reference	Flow rate	Extraction time	Mass of raw material (g)
Baysal et al. (2000)	33.33, 66.67, 133.33 g/min	60, 120, 180 min	53
Cadoni et al. (2000)	500 mL/min	30 min	2.5
Ciurlia et al. (2009)	133.33–333.33 g/min	480 min	3,000
De la Fuente et al. (2006)	–	720 min equilibration period	0.2
Del Castillo et al. (2003)	–	120 min	20
Egydio et al. (2010)	0.85 g/min, 1.7 g/min	180 min, 360 min	15
Filho et al. (2008)	4.08 g/min	10 min static period 120 min	5.6
Guedes et al. (2013)	2, 4.3 g/min	240 min	0.4
Gomez-Prieto et al. (2003)	4 mL/min	30 min	0.5
Nobre et al. (2009)	0.26, 0.59, 1.18 g/min	–	1.5
Ollanketo et al. (2001)	1.5 mL/min	5, 12, 19, 26, 38, 50, 62, 79 min	0.3

(continued)

Table 11.3 (continued)

Reference	Flow rate	Extraction time	Mass of raw material (g)
Rozzi et al. (2002)	2.5–15 mL/min	20 min	3
Sabio et al. (2003)	13.2, 22.5 g/min	–	40–50
Machmudah et al. (2012)	2–4 mL/min	180 min	4
Saldana et al. (2010)	0.5, 1.2 mL/min	360 min	10
Shi et al. (2009a)	1.5 mL/min	90 min	5
Shi et al. (2009b)	3,500 mL/min	–	0.5
Topal et al. (2006)	1.5–4.5 mL/min	330 min	–
Vagi et al. (2007)	–	–	1,000
Vasapollo et al. (2004)	133.33–333.33 g/min	120–480 min	3,000
Vaughn et al. (2008)	1.5 mL/min	5 min static extraction, 30 min dynamic extraction	0.5
Machmudah et al. (2006)	2–4 mL/min	240 min	7
Yi et al. (2009)	1.0, 1.5, 2.0 mL/min	90 min	–

In the supercritical CO_2 extraction of lycopene from tomatoes, the relationship between the difference in moisture content and temperature may be of importance for maximizing lycopene yields; in the case of lycopene extraction from watermelon it was observed that aside from the storage period, the moisture content of the watermelon material to be extracted also played a role in the extraction process (Vaughn et al. 2008).

In a study conducted on the supercritical CO_2 extraction of *trans*-lycopene, Nobre et al. (Konar et al. 2012) utilized fresh tomato industrial waste which was composed of a mixture of skins and seeds with an initial moisture content of 82.9 %. The tomato industrial waste was then dried to moisture contents of (58.1, 22.8, 4.6) %, respectively. When Soxhlet extraction was carried out on the fresh tomato industrial wastes as well as on the dried samples, the fresh material showed the highest amount of *trans*-lycopene present, 691 $\mu g/g_{\text{oil-free dry matter}}$, while the dried samples with moisture contents of (58.1, 22.8) %, showed a *trans*-lycopene content of (560 and 578) $\mu g/g_{\text{oil-free dry matter}}$ respectively, which was only slightly lower than the fresh material. The sample with the lowest moisture content of 4.6 % contained 52.1 $\mu g/g_{\text{oil-free dry matter}}$ of *trans*-lycopene, which was the lowest amount present. The authors of the study attributed these results to an explanation provided by Brunner (Doker et al. 2004)—namely, that in the dried samples, the lipid pillars in the plant cell elementary membrane close due to lack of water, making the membrane impermeable. This means that the lack of water content essentially

changes the structure of the tomato skins and makes it impermeable; lycopene is hence trapped by the tomato skin structure which hinders its extraction by supercritical CO_2. A high performance liquid chromatography (HPLC) analysis of the Soxhlet extracts of the dried samples detected no lycopene degradation products; thus it was proved that lycopene did not undergo degradation but was simply unavailable for extraction.

In the supercritical CO_2 extraction of carotenoids from carrot it was observed that the presence of a large amount of water in the carrot matrix increased the path lengths through which the carotenoids had to cross to reach the supercritical CO_2. Furthermore, these longer path lengths were filled with water, which is a relatively polar compound, increasing the diffusion resistance of the carotenoids and thus decreasing extraction efficiency (Sun and Temelli 2006).

Obviously, for non-polar compounds such as carotenoids, the presence of water within the sample can inhibit the extraction of the target compound by supercritical CO_2 (Brunner 1994). For relatively polar compounds, however, water can act as a co-solvent in the extraction process. Furthermore, water may also have a positive effect by swelling the sample matrix, thus making it easier for the supercritical CO_2 to penetrate and diffuse into the sample. This positive effect, however, is compromised by water's more negative effects on extraction efficiency. These observations might help explaining the results obtained by Nobre et al. (2009). A similar situation also probably occurred when Vasapollo et al. (2004) attempted the extraction of lycopene from sun-dried tomatoes containing (50–60) % moisture with supercritical CO_2 at different pressures and temperatures. They found that only traces of lycopene could be extracted and attributed the results to the high content of moisture present in the tomatoes.

In a study on the supercritical CO_2 extraction of lycopene from tomato juice, Egydio et al. (2010) removed the water in the tomato juice pulp by one to four successive washes with 6 mL 99.5 % ethanol at room temperature, with centrifugation after each washing step. Extraction was carried out on the tomato juice sample at 60 °C and 27.5 MPa, and it was found that after one ethanolic washing step, no lycopene was recovered. They attributed the result to the large amount of water still present in the sample. It was also found that the extraction bed was compressed and that no supercritical CO_2 flux through the extraction bed was noticed, due to the low solubility of supercritical CO_2 in water. Reverchon and De Marco (2006) suggested that water content in a sample matrix, along with extractable liquid compounds, can cause sample particles to coalesce, which can result in irregular extraction along the extraction bed, a phenomenon also known as channeling.

11.3.1.8 Effect of Feed Composition

Composition of the feed has the most significant effect on the extraction of antioxidant activity (Louli et al. 2004). In a work done by Nobre et al. (2009), two samples of tomato industrial waste, with different *trans*-lycopene content, were

submitted to SFE at 60 °C and 30 MPa. It was reported that when the feed had a lower content of *trans*-lycopene, the recovery was faster and higher. In addition, when the starting material had a higher content of *trans*-lycopene, the solvent/feed ratio increased i.e. the amount of CO_2 required for the extraction was higher for the same flow-rate, and the increase in the extraction time resulted in degradation of the *trans*-lycopene in the extraction cell, thus lowering its recovery.

11.3.1.9 Effect of Pretreatment

Differences in the quality have been observed between the extracts obtained from samples of different origins and samples stored at air-dried and frozen conditions. For maximum recovery of high valued products, the by-products must be stored at frozen condition rather than at air-dried state. Presence of water in plant tissue interferes with the effectiveness of SFE as it hinders the diffusion of the supercritical CO_2 into the solid matrix. Thus, different techniques, such as oven-drying, freeze-drying or using adsorbents have been investigated for removing water from the sample. It has been reported that the extraction yield of freeze-dried samples was higher than that of oven-dried samples (Louli et al. 2004).

In a work done by Louli et al. (2004), the effect of feed pretreatment (crushing) on the supercritical extraction of the antioxidants from red grapes pomace was investigated and it was reported that crushing the pomace resulted in a small increase of the extracted compounds. Crushing was therefore not considered as a decisive parameter for increasing the efficiency of the process.

According to Mukhopadhyay and Karamta (2008), pretreatment of washing the ground feed with water-soluble organic solvent and subsequent extraction with the same solvent facilitated the easy removal of bound water to get dehydrated feed and also resulted in enrichment by removal of undesirable constituents from the dehydrated feed. The lycopene content in the extract improved from 2.7 % (from dehydrated feed) to 4.5 % (from enriched feed). SFE cum-fractionation from such enriched feed resulted in efficient recovery of active ingredients with high purity within 2–5 h at (30–45) MPa and (60–70) °C.

Kha et al. (2014) reported that enzymatic pretreatment of feed resulted in lower β-carotene and lycopene content in the extracts due to larger contacting surface area of enzymatic-treated feed with oxygen that caused carotenoids oxidation.

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Chapter 12
Lipid Processing and Lipase Activity Under High Pressure Conditions

Luis Vázquez and Carlos F. Torres

12.1 Introduction

Increased understanding of the nutritional values of lipids has led to the development of novel technologies for modifying fats and oils, or enrichment and isolation of bioactive lipophilic compounds to enhance the health benefits resulting from ingestion of these substances. In the last decade, supercritical fluids more and more have been proved as environmentally benign media for extraction, chemical and enzymatic reactions and related processes. Many new processes and products have been developed, using the inherent physical and chemical properties of supercritical fluids.

In this chapter supercritical fluid extraction (SFE) of fat and lipid compounds is reviewed. The effect of pre-treatment and additional conditioning processes on SFE processing is discussed. Finally, supercritical CO_2 biocatalytic strategies focusing on the production of bioactive lipids for food industry are presented.

12.2 Supercritical Fluid Extraction of Lipid Compounds

The widest application of supercritical fluids is extraction (SFE). This technology takes advantage of the solvent properties of a fluid near its critical point. Supercritical fluids have physicochemical properties intermediate between those of liquids and gases (Rizvi et al. 1986). These properties, chiefly density, can be altered by varying the pressure and temperature. Thus, certain selectivity for a

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specific compound can be conferred by the solvent. The use of supercritical fluids is one possibility to carry out chemical technologies in a sustainable manner ("*green chemistry*"). Among the green solvents used in SFE, carbon dioxide is undoubtedly the most commonly employed, providing several advantages compared to other solvents. CO_2 is widely used due primarily to its low critical parameters (31.1 °C, 73.8 bar), low cost, nontoxicity, chemical inertness, and nonflammability (Hauthal 2001; Rozzi and Singh 2002). CO_2 is environmentally friendly and generally recognized as safe (GRAS). Supercritical CO_2 ($SCCO_2$) is also attractive because of its high diffusivity combined with its easily tunable solvent strength. Another advantage is that CO_2 is gaseous at room temperature and pressure, which makes extract recovery very simple and provides solvent-free extracts. Also important for food and natural products is the ability of SFE using CO_2 to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds (Schütz 2007). These characteristics are extremely important for applications of this technology in the food processing industry. Due to the absence of toxic residue in the final product, among other advantages, $SCCO_2$ is especially useful for extraction in two situations: (a) extracting valuable bioactive compounds such as flavors, colorants, and other biomolecules or (b) removing undesirable compounds such as organic pollutants, toxins, and pesticides (Pereira and Meireles 2010).

$SCCO_2$ has a low polarity (with a low solubility parameter, around 15 $MPa^{1/2}$). Its polarity is classifiable in between that of dichloromethane and that of ethyl ether. Therefore, its efficiency to extract polar compounds from natural matrices is quite limited. To overcome this problem, polar co-solvents (methanol, ethanol, water) are commonly used in small amounts to increase the solubility of polar compounds in the supercritical mixture (Mendiola et al. 2013). On the other hand, consequently, it is highly selective and dissolve quite readily non-polar lipophilic compounds like fats, oils and aroma components, such as terpenes (Hierro and Santa-María 1992).

For that reason, numerous SFE methods have been developed to extract lipid and lipid soluble materials from complex sources, including (but not limited to) the extraction of lipid-soluble vitamins (A, D, E, and K), various seed, nut, bean, and wood oils, essential oils, total meat fats, phospholipids, pesticides, cholesterol, and pharmaceutical components (Berg et al. 1997; Gonzalez-Vila et al. 2000; Hopper and King 1991; King et al. 2001a; Ronyai et al. 1998; Sovova et al. 2001). In this field, Sahena et al. (Sahena et al. 2009) reported a new complete review describing the main aspects and applications of supercritical CO_2 in lipid extraction.

Often, SFE methods are preferred over conventional methods because the resulting products are free from organic solvent residues, and there is a minimal risk of thermal oxidation (Astaire et al. 2003). Most of the work published on supercritical fluid extraction in the food processing industry has dealt with applications to extract vegetable oils (Bulley et al. 1984; Christianson et al. 1984; Daković et al. 1989; Lee et al. 1986; List et al. 1984a, b; Stahl et al. 1980; Taniguchi et al. 1985).

Extraction and fractionation of fats and oils, either from animal or vegetal sources, represent a wide area of application for $SCCO_2$, where it shows remarkable advantages in contrast to conventional technologies. Hence, Mangold reported

some advantages of the $SCCO_2$ extraction vs. extraction with organic solvents, in terms of quality of the oil obtained: oils with less amounts of phospholipids and glycolipids; less content of gossypol; more deodorized and clearer oils; less use of sodium hydroxide and lower losses in the refination step (Mangold 1983).

Brunner reported that the separation of free fatty acids (FFA) by vacuum distillation is efficient; however by using $SCCO_2$ it can be performed at much lower temperatures (Brunner 2000). This fact can play an important role avoiding degradation reactions, such as *cis–trans* isomerization, polymerization or oxidation of the oil. Molero Gómez and Martínez de la Ossa compared the extraction of wheat germ oil with $SCCO_2$ and hexane, concluding that the extraction process using CO_2 could be economically competitive with the conventional process, since it considerably simplifies the oil refinement stages and completely eliminates the solvent distillation steps in terms of consumption (Molero Gómez and Martínez de la Ossa 2000). Likewise, Friederich and List compared the extraction of soybean oil with $SCCO_2$ and hexane, obtaining similar recoveries (around 20 %) and identical fatty acid profiles (Friedrich and List 1982). Cheung et al. obtained significantly higher amounts of ω-3 polyunsaturated fatty acids (PUFA) after SFE compared to Soxhlet extraction (Cheung et al. 1998).

As stated before, due to the mild extraction conditions and the O_2 free ambient involved, SFE with CO_2 is suitable for extraction of non-polar or lipophilic compounds, particularly those that are susceptible to thermal degradation such as lipid antioxidants (Díaz-Reinoso et al. 2006). However, the oxidative stability of walnut oil (Crowe et al. 2002; Crowe and White 2003a, b) and sunflower oil (Calvo et al. 1994) extracted with $SCCO_2$ can be lower than that obtained by using physical methods. These differences can be explained by the amounts of O_2 dissolved in the $SCCO_2$. Also, the oxidative stability is much lower in SFE refined oils than in raw oils, since the amount of tocopherols decreased after refining. However, this problem can be overcome by the addition of trace amounts of ascorbic acid or other antioxidants (Calvo et al. 1994).

Some SFE applications for lipid compounds from different raw materials, with their corresponding conditions of temperature and pressure, are shown in Table 12.1.

12.3 Pre-treatments and Additional Processes

Despite the benefits and flexibility of supercritical and near-critical solvent systems in bioprocessing, it is advantageous, and in many cases necessary, to carry out additional processing operations either before or during supercritical fluid processing and to consider it as a whole integrated process.

Recently, Cathpole et al. (2012a) have investigated the use of pre-treatment processes to increase the yield of bioactives in the feed material by reducing the amount of non-extractable material. These processes include enzyme pre-processing (using proteases, carbohydrases) and acid-base treatments. Pre-processing by

Table 12.1 Summary of some SFE applications for lipids

Raw material	Compounds of interest	*P* (MPa)	*T* (°C)	Ref.
Milk	Fat	10.0–35.0	50–70	Arul et al. (1987)
Milk	Fat	6.9–24.1	40–80	Lim and Rizvi (1995)
Egg	Lipids and cholesterol	31.0	45	Froning et al. (1998)
Beef	Lipids and cholesterol	10.3–31.0	30–50	Chao et al. (1991)
Beef	Lipids and cholesterol	10.3–27.6	40	Merkle and Larick (1995)
Pork	Lipids	10.0–31.0	30–50	Chao et al. (1991)
Schizochytrium limacinum	Lipids	35	40	Tang et al. (2011)
Kalahari melon seed	Phytosterol-enriched oil	30	40	Nyam et al. (2011)
Piquillo red pepper	Vitamin E y provitamin A	24	60	Romo-Hualde et al. (2011)
Corn bran	Ferulatephytoserol ester	13.8–69	40–80	Taylor et al. (2000)
Brown seaweed	Fatty acid composition	24.1–37.9	40–50	Cheung et al. (1998)
Cardamom seed	Fatty acid composition	10–30	35–55	Hamdan et al. (2008)
Cotton seed	Fatty acid composition	51.7–62	100	Taylor et al. (1997)
Cunninghamella echinulata	Fatty acid composition	20–35	40–70	Certik and Horenitzky (1999)
Ground beef	Fatty acid composition	38/80	80	King et al. (1996)
Grape seeds	Fatty acid composition	35/40	40	Molero et al. (1995)
Mackerel	Fatty acid composition	20.7–34.5	35–55	Temelli et al. (1995)
Palm kernel	Fatty acid composition	20.7–48.3	40–80	Zaidul et al. (2007)
Pecan	Fatty acid composition	41.3–66.8	45–74	Alexander et al. (1997)
Pistachio	Fatty acid composition	10–15	40-80	Sheibani and Ghaziaskar (2008)
Peanuts	Fatty acid composition	13.8–55.2	80	Chiou et al. (1996)

(continued)

Table 12.1 (continued)

Raw material	Compounds of interest	*P* (MPa)	*T* (°C)	Ref.
Pythium irregulare	Fatty acid composition	13.7–27.5	40–60	Walker et al. (1999)
Rice bran	Fatty acid composition	17–31	0-60	Shen et al. (1996)
Rice bran	Fatty acid composition	13.6–20.5	40–90	Dunford and King (2001)
Rosehip seed	Fatty acid composition	15–45	40–80	Machmudah et al. (2007)
Safflower	Fatty acid composition	51.7–62	100	Taylor et al. (1997)
Sardine oil	Fatty acid composition	9.6–19.5	40–80	Riha and Brunner (2000)
Sea buckthorn	Fatty acid composition	27.6	34.5	Xu et al. (2008)
Soybean	Fatty acid composition	51.7–62	100	Taylor et al. (1997)
Sunflower	Fatty acid composition	51.7–62	100	Taylor et al. (1997)
Wheat germ	Fatty acid composition	20–35	40–60	Shao et al. (2008)
Rapeseed	Fatty acid composition, tocopherol content	51.7	100	Bruhl and Matthaus (1999)
Oat bran	Digalactosyldiacylglycerols	40	50–70	Andersson et al. (1997)
Boletus edulis	Fatty acids	35	40	Vidovic et al. (2011)
Borago officinalis	Fatty acids	35	65	Ramandi et al. (2011)
Peach kernels	Fatty acids	30	50	Mezzomo et al. (2010)
Broccoli leaves	Fatty acids	30	60	Arnáiz et al. (2011)
Hemp seeds	Fatty acids	30	40	Da Porto et al. (2012)
Camellia sinensis	Fatty acids and antioxidants	32	45	Wang et al. (2011)
Sesame	Fatty acids	20	35	Carvalho et al. (2012)
Brazilian red-spotted shrimp waste (shell and tail)	ω-3 PUFA, astaxanthin	30	50	Sanchez-Camargo et al. (2012)

(continued)

Table 12.1 (continued)

Raw material	Compounds of interest	*P* (MPa)	*T* (°C)	Ref.
Brazilian red-spotted shrimp waste (shell and tail)	ω-3 PUFA, astaxanthin	30	50	Sanchez-Camargo et al. (2011)
Fish by-product (Indian mackerel skin)	ω-3 PUFA	35	75	Sahena et al. (2010)
Fish by-products (off cuts from hake, orange roughy and salmon, and livers from jumbo squid)	ω-3 PUFA	25	40	Rubio-Rodriguez et al. (2010)
Fish by-products (trout heads, spines and viscera)	ω-3 PUFA	50	60	Fiori et al. (2012)
Fish oil (*Pseudoplatystoma corruscans*)	ω-3 PUFA	20	33–40	Lopes et al. (2012)
Northern shrimp byproducts (heads, shell and tail)	ω-3 PUFA	35	40	Treyvaud Amiguet et al. (2012)
Sea urchin gonad	ω-3 PUFA	28	50	Zhu et al. (2010)
Shellfish by-products (Abalone gonad)	ω-3 PUFA	28	50	Zhou et al. (2012)
Pomegranate seed oil	Fatty acids, tocopherols	15–30	35–65	Liu et al. (2012)
Vinification residues	Fatty acids, α-tocopherol	25	80	Agostini et al. (2012)
Wheat bran	Tocopherols	30	60	Kwon et al. (2010)
Rice bran	α-Tocopherol	48.2–62	70–100	Shen et al. (1997)
Rice germ	Tocols	13.8	60	Ko et al. (2012)
Sea buckthorn (*Hippophae rhamnoids*)	Tocopherols, lycopene and β-carotene	40	35	Kagliwal et al. (2011)
Carrot	Carotenes	34.2–57.0	30–50	Vega et al. (1996)
Alfalfa leaf	Carotene and lutein	10.0–70.0	40	Favati et al. (1988)
Sweet potato	β-carotene	13.8–41.4	38–48	Spanos et al. (1993)
Bixa orellana seeds	Bixin	20.7–48.3	40–55	Degnan et al. (1991)
Tomato paste waste	β-Carotene and lycopene	20.0–30.0	35–65	Baysal et al. (2000)
Tomato skin	Lycopene	40.5	60–110	Ollanketo et al. (2001)

(continued)

Table 12.1 (continued)

Raw material	Compounds of interest	*P* (MPa)	*T* (°C)	Ref.
Guava by-products	Lycopene	30	55	Kong et al. (2010)
Tomato juice	Lycopene	35	40	Egydio et al. (2010)
Tomato peel by-products	Lycopene	40	90	Machmudah et al. (2012)
Red-ripe tomato cultivars	Lycopene	45	65–70	Lenucci et al. (2010))
Spinach	Lutein	30	50	Chen et al. (2012)
Chlorella vulgaris	Lutein	40	40	Ruen-ngam et al. (2012)
Scenedesmus almeriensis	Lutein and β-carotene	40	60	Macias-Sanchez et al. (2010)
Hemerocallis disticha	Lutein, zeaxanthin	60	80	Hsu et al. (2011)
Chlorella vulgaris	Canthaxanthin and astaxanthin	30	40	Coelho et al. (2012)
Haematococcus pluvialis	Astaxanthin	40	70	Krichnavaruk et al. (2008)
Monoraphidium sp. *GK12*	Astaxanthin	20	30	Fujii (2012)
Nannochloropsis oculata	Lipids, zeaxanthin	35	50	Liau et al. (2010)
Oil from bitter orange peel	Terpens	7.7–12.0	40	Chouchi et al. (1996)
Juice from citrus fruit	Limonin	20.7–41.4	30–60	Kimball (1987)
Dried orange peel	Essential oils	10.0–28.0	40–50	Blasco et al. (1999)
Aromatic plants	Essential oil	20.0	40	Blasco et al. (1999)
Lamiaceae plants	Essential oil	30	40	Fornari et al. (2012)
Salvia officinalis	Essential oil	30	40	Micic et al. (2011)
Spearmint (*Mentha spicata*)	Essential oil	9	35	Ansari and Goodarznia (2012)
Spearmint (*Mentha spicata*)	Essential oil	30	50	Almeida et al. (2012)
Rapeseed	Essential oil	30	50	Yu et al. (2012)

(continued)

Table 12.1 (continued)

Raw material	Compounds of interest	*P* (MPa)	*T* (°C)	Ref.
Rizhomes of Cyperus	Essential oil	30	35	Wang et al. (2012)
Lippiadulcis	Hernandulcin and other sesquiterpenes	12	35	de Oliveira et al. (2012)
Mushrooms	Oleoresins	11.5–14.9		Del Valle and Aguilera (1989)
Hops	Humulone, lupulone and essential oils	20.0	40	Langezaal et al. (1990)
Eucalyptus leaves	Oil with high antioxidant activity	20.0	50	Fadel et al. (1999)
Fish oils	Squalene	20–30	60	Catchpole et al. (2000)
Terminalia catappa leaves	Squalene	30	40	Ko et al. (2002)
Amaranth grains	Squalene	20	50	He et al. (2002, 2003)
Olive oil deodorized distillate	Squalene	18	70	Vázquez et al. (2007)

Source: Modified from Mendiola et al. (2013) and Sahena et al. (2009)

chemical or enzymatic reactions can be an effective way to facilitate the extraction, for example when the lipids are strongly bound to cellular structures.

An exemplar process was carried out by Billakanti et al. (2013a) for the pretreatment of a macro-algae, *Undaria pinnatifida* using an enzyme, followed by extraction of fucoxanthin using either ethanol or dimethyl ether. *Undaria pinnatifida* produces the valuable lipophilic pigment fucoxanthin and also a small amount of lipids rich in Stearidonic Acid (SDA) (C18:4 ω-3) and EPA. The highest yields of fucoxanthin (94 %) and lipids (94 %) rich in PUFA were obtained from fresh (wet) *U. pinnatifida* by enzyme pre-processing, followed by extraction using dimethyl ether with ethanol as a co-solvent. Enzyme pre-processing using alginate lyase resulted in the hydrolysis of cell wall polysaccharides, resulting in high extraction yields. The hydrolysis time, pH and temperature were found to be the most important parameters for the enzyme pre-processing step and for minimizing fucoxanthin losses due to oxidative degradation.

Enzymes can also be used to convert lipids to free fatty acid or ester forms to make them more easily extractable, or to cleave selected fatty acids and enable fractionation of fatty acids and transesterification reactions to be carried out. Some compounds of interest can also be processed more effectively in a different form, for example omega-3 fatty acids must be separated from the glycerol backbone if they are to be processed individually and ester forms of fatty acids are generally easier to solubilize. These processes can lend themselves to supercritical extraction either post reaction, or even *in situ* (Catchpole et al. 2012a).

Position specific enzymes have a high regiospecificity for the sn-1 and sn-3 positions of the glycerol backbone, which allows the enrichment of PUFA in the sn-2 position either by hydrolysis (Linder et al. 2002) or by transesterification processes (Haraldsson et al. 1997; Muñio et al. 2008). By adjusting extraction conditions, $SCCO_2$ allows selective removal of the FFA or fatty acid ethyl esters (FAEE) produced in the enrichment enzymatic reaction, leaving a PUFA enriched partial glyceride fraction in the raffinate. Besides, lipases can present activity only for specific fatty acids, and in general, they act weakly on PUFA (Shimada et al. 1997). Thus, PUFA can also be enriched in the undigested glyceride fraction by selective hydrolysis of PUFA-containing oil with a lipase (Shimada et al. 1998). This pretreatment facilitates the subsequent separation of the PUFA-containing glycerides and FFA by supercritical fluid extraction.

Vázquez et al. (2006) studied the recovery of minor lipid compounds (tocopherols and phytosterols) from sunflower oil deodorizer distillates using countercurrent $SCCO_2$ extraction. Since the raw material employed contained large amounts of triacylglycerols (TAG) and FFA, chemical transformation of these compounds into their corresponding FAEE was previously carried out, in order to favor the concentration of minor lipids in the raffinate product. Extractions of the original and pretreated raw material were carried out in a pilot-scale plant at 65 °C, with pressures ranging from 15 to 23 MPa and solvent-to-feed ratios from 15 to 30. The chemical transformation of the deodorizer distillate composition significantly enhances the concentration of minor lipids in the raffinate product.

These pretreatments also can play an important role for concentration of bioactive ether lipids, also known as alkylglycerols, from natural sources, such as shark liver oil. Isolation and purification of ether lipids from natural sources are difficult, mainly because of the presence of TAG, which have analogous structure, molecular weight, polarity and volatility. In these terms, ether lipids and TAG are similar molecules and their separation by SFE or other technologies can be unfeasible since these compounds would have similar behavior. Recently, Vázquez et al. (2008) have been able to concentrate ether lipids by a two-step methodology based on the transesterification by ethanolysis of shark liver oil and subsequent SFE to purify the products. The transesterification reaction converts triacylglycerols and di-esterified alkylglycerols into the corresponding FAEE, plus non-esterified alkylglycerols together with minor amounts of MAGE and lower glycerides. Saponification has also been used to modify the original shark liver oil (Torres et al. 2007). As stated previously, saponification or ethanolysis reactions are necessary because of the presence of TAG that could interfere in the alkylglycerol fractionation.

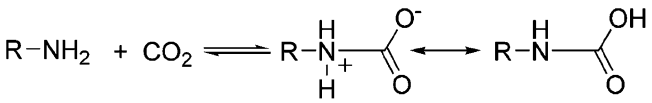
Another type of integrated or "in situ" processes includes adsorption or reaction operations that are carried out under supercritical (or near-critical) solvent conditions. The supercritical fluid solvent acts as a high mass and heat transfer solvent system with controllable solvent density ideal for many adsorption/desorption operations. In the case of enzymatic processing, continuous processing is possible and continuous removal of reaction products gives potential for more complete reaction to occur (Catchpole et al. 2012a).

Introducing adsorbents into a supercritical fluid extraction system is an alternative attractive method to improve the selectivity. Adsorption phenomena with supercritical fluids have been studied in the context of using the adsorbent as a separating agent or in the context of using the supercritical fluid to regenerate the adsorbent. A separation process in the presence of an adsorbent is expected to be strongly influenced by the proximity of the experiment to the fluid´s critical point. King and List (1996) reported an unusually strong pressure dependence for adsorption in mixtures at supercritical conditions.

Separation of two or more solutes utilizing adsorption and desorption behavior in $SCCO_2$ have been reported. Lim and Rizvi (1996) evaluated the adsorptive separation process for cholesterol reduction in anhydrous milk fat by $SCCO_2$ using magnesium silicate. Adsorbents were also used for citrus oil processing. Hence, Barth et al. (1994) and Chouchi et al. (1995, 1996) used $SCCO_2$ to desorb or extract the oxygenated aroma compounds after the equilibrium saturation between a feed and an adsorbent under ambient conditions in a batch operation. They obtained a high-quality essential oil containing less terpenes and less nonvolatile compounds by $SCCO_2$ desorption with increasing pressure. However, high pressure or cosolvent was required to regenerate the adsorber because the nonvolatile compounds, such as waxes and pigments, were more strongly adsorbed on silica gel than aroma compounds. Sato et al. (1998) fractionated citrus oil by pressure swing adsorption in $SCCO_2$. They used a continuous pressure swing operation between the adsorption step at 8.8 MPa and 313 K and the desorption step at 19.4 MPa and 313 K, including a rinse step. Highly concentrated fraction of oxygenated compounds was continuously obtained for the desorption step and the blowdown step (depressurization). These authors also used the methodology mentioned to separate α-tocopherol and squalene from a mixture of these compounds. In this study, the pressure swing operation between the adsorption step at a lower pressure, and the desorption step at a higher pressure was performed with octadecylsilica as adsorbent. Thus, α-tocopherol was concentrated from 20 wt% of tocopherol in feed mixture to 60 wt% in product in the desorption step, and squalene was also concentrated from 80 wt% in feed mixture up to 98 wt% in product in the adsorption step (Wang et al. 2004).

12.4 Biocatalysis of Lipid Compounds in $SCCO_2$

Stricter environmental laws related to the use of organic solvents in many areas of the fats and oils industry have stimulated the search for sustainable technologies for lipid processing. Furthermore, as consumers demand “natural” products, the use of potentially toxic solvents in various production processes is being more and more constrained. The application of pressurized carbon dioxide in lipid processing may offer new opportunities to reduce the amount of organic solvents needed. It has been almost three decades since the first reports of enzyme-catalyzed reactions in supercritical fluids (SCFs) were published. Randolph et al. (1985) and Hammond



Scheme 12.1 Reversible carbamate formation between CO_2 and lisyne residues on the surface of an enzyme

et al. (1985) both used enzymes as simple suspensions in SCFs. CO_2 tends to be the SCF of choice for biocatalysis because it is cheap, readily available, and considered the most "green" of the SCFs with suitable critical parameters that are compatible with conditions required for enzymatic reactions.

12.4.1 Enzymatic Activity in $SCCO_2$

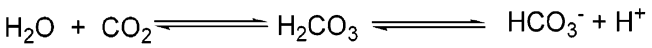
Carbon dioxide is involved in two chemical processes that have the potential to reduce or destroy the catalytic activity of an enzyme. These are the formation of carbamates between CO_2 and lysine residues on the surface of the enzyme (Scheme 12.1) and the formation of carbonic acid by reaction between CO_2 and any water present in the system (Scheme 12.2) (Hobbs and Thomas 2007).

Some reports have suggested that carbamate formation is advantageous as this can result in enhanced stereoselectivity of a reaction (Ikushima et al. 1995). On the other hand, some publications claim that carbamates are the cause of enzyme inactivation in $SCCO_2$ (Habulin and Knez 2001) either through blocking the active site or causing a detrimental conformational change in the enzyme, and so, an alternative reaction medium, supercritical or otherwise, may provide a better solution.

The second property of CO_2 that can be detrimental to enzyme activity is the lowering in pH of water present in $SCCO_2$ (Kamat et al. 1995). In nonaqueous media, enzymes can change their catalytic activity if the pH of the microaqueous environment around them is altered. CO_2 can dissolve in the hydration layer associated with the enzyme, thereby altering the local pH by formation of carbonic acid by reaction between CO_2 and any water present (Scheme 12.2) and, hence, affecting enzyme activity. As a solution, the addition of organic and inorganic buffers to the water/$SCCO_2$ microemulsion droplets results in an increase in pH from 3 to values of 5–7 (Holmes et al. 1999).

A number of studies on the effect of changes in pressure on enzyme-catalyzed reactions have been reported, and it has been indicated that the changes in enantioselectivity with pressure are due to density of $SCCO_2$ changes, the interaction of CO_2 and enzyme molecules, and water content (Glowacz et al. 1996).

For green reactions, it is important that the enzyme can be easily recycled and that it will retain its activity over many reaction cycles; hence, the enzyme needs to be stable to many pressurization and depressurization cycles. Cooling of $SCCO_2$



Scheme 12.2 Formation of carbonic acid and its dissociation to the bicarbonate anion in $SCCO_2$

under the critical point before depressurization or depressurization from the liquid phase should be avoided to minimize enzyme inactivation (Gießauf et al. 1999).

Another interesting feature is the treatment of enzymatic preparations with dry and humid $SCCO_2$ for impurities removal and improvement of enzymatic activity. It was found out that the preparations treated are significantly more active with the long chain triglyceride triolein (675 % residual activity with 100 μL of water added), while there is a loss of activity towards the short chain triglycerides, tributyrin and triacetin. Treatment with humid $SCCO_2$ activates the PPL that is active towards long chain triglycerides and denatures the other hydrolases that are active only towards short chain triglycerides (Bauer et al. 2001).

12.4.2 Enzymatic Reactions under High Pressure Conditions

One of the most interesting properties of biocatalysis is enzyme selectivity. The reaction medium can be utilized as a tool to improve this feature of some enzymes. As an example, enzymatic hydrolysis of conjugated linoleic acid-enriched anhydrous milk fat using $SCCO_2$ has been shown to be an efficient method to obtain FFA, reaching a maximum of 86.79 ± 7.28 % (w/w) in 2 h at 55 °C, 23 MPa and a fat:water ratio of 1:5 (mol/mol), when Lipozyme TL IM was utilized as a catalyst. Approximately 98 % of CLA in TAG form was converted to FFA form at 55 °C, 30 MPa and a fat:water ratio of 1:30 (mol/mol), when Lipozyme TL IM was utilized as a catalyst. The presence of intermediate products (monoacylglycerol, MAG; and diacylglycerol, DAG) and TAG indicates that the hydrolysis was not complete for all the conditions studied. The findings may contribute to the development of new approaches for the isolation of CLA. In addition, hydrolyzed products obtained might find applications in food and personal care products (Prado et al. 2012).

The application of an $SCCO_2$/lipase based reaction has been also used to synthesize sterol esters which have utility as functional food ingredients. The feasibility of conducting lipase-catalyzed reactions between sterols/stanols and various *n*-alkanoic acids was surveyed in this study. Specifically, several lipases were evaluated with respect to their ability to form long chain esters with a model sterol and stanol, respectively, under $SCCO_2$ conditions. Syntheses were conducted under both static and dynamic flow conditions using a micro reactor approach to expedite experimentation and minimize the expense of costly reagents. Reaction yields were optimized with respect to pressure, temperature, and flow rates (King et al. 2001b).

Dipalmitin has been also synthesized from palmitic acid and glycerol in $SCCO_2$ with an immobilized enzyme catalyst. The optimum reaction conditions, such as

reaction time, temperature, enzyme loading, substrate molar ratio, and pressure, were determined. Reaction in organic solvent (tert-butanol) system was also investigated, and the highest conversion of palmitic acid and highest yield of dipalmitin were obtained in the $SCCO_2$ with an extremely low enzyme concentration, 0.34 g/L. A phase behavior study indicated that the solubility of monoglyceride in supercritical carbon dioxide was much higher than that of diglyceride. It can be inferred that the difference in the solubility of the products favors production of diglyceride. A phase transfer model was proposed to illustrate the highly selective synthesis process for diglyceride in $SCCO_2$. When monoglyceride is synthesized as the initial product, it immediately dissolves into the supercritical carbon dioxide, which promotes dissolution of palmitic acid in $SCCO_2$. The advantages of $SCCO_2$ such as low viscosity and high diffusivity reduce the mass transfer resistance and make it easier for the monoglyceride to further react with the fatty acid. Thus, the reaction rate and material conversion rate are enhanced by formation of the homogeneous phase. However, once the diglyceride is generated, it immediately leaves the homogeneous phase due to its poor solubility in $SCCO_2$, and a two-phase system forms. This particular phase behavior lowers the probability of diglyceride reacting with the acid and in time terminates the reaction. Thus, the high selectivity for diglyceride shown above is achieved because of the solubility difference between the various products (Tao et al. 2013).

12.4.3 Reaction Medium Expanded by CO_2

Historically, emphasis has been put on performing biocatalysis in $SCCO_2$ in supercritical conditions of the whole reaction bulk. However, the major drawback of enzymatic reactions in $SCCO_2$ carried out in a single supercritical phase is the high pressures (on the order of hundreds of bars) required to ensure entire solubility of many organic compounds in CO_2. A thumbnail sketch of advantages of using CO_2 expanded reaction media (i.e. reaction media where $SCCO_2$ is used as solvent although the reaction is carried out in the liquid reaction bulk in subcritical conditions) is herein offered.

Nowadays, only few examples of catalytic reactions carried out in subcritical conditions have been reported. Moreover, performing biocatalysis in CO_2 expanded reaction media, may offers several advantages as: (a) substantial reduction in viscosity and enhancement in diffusivity of the reaction media, minimizing the transport limitations towards the catalyst; (b) easily tunable solvating power, as well as in supercritical conditions (Huang et al. 1991); (c) higher substrates concentration in the enzyme phase; (d) lower process pressures (tens of bars) than reactions in supercritical conditions (hundreds of bars); (e) pressure-tunable dielectric constants. On this basis, CO_2 expanded reaction media open a new way towards environmentally benign esterification catalyst systems for such important chemical reactions.

The esterification of oleic acid by 1-octanol catalyzed by Lipozyme RM IM has been investigated in different media. CO_2 expanded reaction mixture resulted in enhancing the reaction kinetics respect to the solvent-free system, requiring lower enzyme concentrations desired at commercial industrial scales (Laudani et al. 2007). Higher fatty acid ester concentration was also obtained in $SCCO_2$ when compared with those attained over *n*-hexane under identical reaction conditions. CO_2 expanded reaction mixture led to higher performance than when diluted in a single supercritical phase, since the reactant mixture was more concentrated in the CO_2 expanded liquid bulk around the catalyst, due to the adsorption of substrates at enzyme surface was related to the limiting rate step. The one-to-two orders of magnitude higher intrinsic diffusivity of substrates in CO_2 expanded reaction bulk reduced the interphase transport limitations, improving hence the reaction performance on the basis that the mass transfer of substrates towards the catalyst was sufficiently fast in biphasic conditions. The outlook for exploitation of biocatalytic reactions in CO_2 expanded media in developing new biotrasformations schemes seems to be highly promising.

Lipids saturated with CO_2 under moderate pressure expand in volume, and their physical properties change substantially. The unique properties of CO_2-expanded lipids and their relevance for promising new applications have been described (Seifried and Temelli 2009). The solubility of triacylglycerols in $SCCO_2$ at moderate temperatures and pressures is relatively low, but it is still possible to benefit from the properties of $SCCO_2$ since as the pressure is increased, CO_2 dissolves in the liquid lipid phase. Lipids saturated with CO_2 under moderate pressure expand in volume and their physical properties, such as viscosity, density, and interfacial tension, change substantially in a way to enhance mass transfer properties. This property has been recently reported for the enzymatic interesterification between canola oil and fully hydrogenated canola oil (Jenab et al. 2013).

Dense CO_2 showed to be a potential medium for the high-pressure catalytic preparation of lauryl oleate by esterification of free fatty acid with 1-dodecanol over immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM). Kinetic observations on the pressure effect exhibited that dense CO_2 expanded reaction mixture in subcritical conditions led to higher performance than when diluted in a single supercritical phase. The potential of developing enzymatic reactions in dense CO_2 expanded liquid bulk in subcritical condition has been explained on the basis of the high solubility of carbon dioxide in substrates liquid mixture. If these phenomena are general, the strategy of using dense CO_2 expanded reaction media in subcritical reaction bulk may find numerous biotechnological applications (Knez et al. 2007).

MAG and DAG have been successfully synthesized from palmitic acid and glycerol in CO_2-expanded acetone using Novozyme 435. Substrate ratio and additional water showed a strong effect on the total conversion and the selectivity to MAG and DAG. Optimum conditions were selected at 85 bar, 50 °C and 25 % of enzyme related to the amount of dissolved palmitic acid (Tai and Brunner 2011).

12.4.4 Other Biocatalytic Strategies

Mori et al. (1998), using a lipid coated enzyme preparation from *Rhizopus delemar*, investigated the enzymatic esterification of lauric acid with glyceride at 40 °C. The lipase is soluble in $SCCO_2$ and the reaction proceeded five to tenfold faster in $SCCO_2$ (200 bar) than in benzene at atmospheric pressure. In addition, the enzyme activity could be switched on and off by only adjusting pressure or temperature of CO_2 media.

The combined use of lipases and supercritical carbon dioxide, to produce two types of milk fat derivatives enriched in short-chain fatty acids via ethanolysis, has been reported (Lubary et al. 2010a, b). These studies investigate the effects of integrating reaction and extraction by the use of $SCCO_2$. In-situ removal of reaction products during reaction provides additional advantages to the overall process: it may enhance reaction selectivity and, in the case of equilibrium reactions, it may increase the production rate due to the equilibrium shift imposed by lowering the product concentrations.

This approach allows the overall process to be tailored towards better overall fractionation, targeting of specific compounds or product properties and utilization of the supercritical fluid solvent state to enhance or create new processing opportunities that could not be realized in conventional solvent systems (Catchpole et al. 2012b).

Currently, the most common way for extraction of carotenoids is by liquid solvent extraction using toluene, hexane, or petroleum ether. However, the conventional method involves the discharge of potentially hazardous solvents to the environment and can also damage the functional properties of the extracts by hydrothermal stress. Therefore, alternative extraction techniques with better selectively and efficiently are sought. Supercritical fluid extraction (SFE) is an alternative separation technology (Roh et al. 2008). The extraction of lipids containing polyunsaturated fatty acids and fucoxanthin using enzyme-assisted dimethylether + ethanol co-solvent extraction was successfully demonstrated at the laboratory-scale, achieving the almost complete extraction of lipids and fucoxanthin (Billakanti et al. 2013b). Unfortunately, enzyme pretreatment combined with supercritical fluid extraction of this type of lipids has not been reported yet.

There has been a growing interest in the biotransformation of natural antioxidants for the design and improvement of nutraceuticals and foods beneficial to health. Esterification of phenolic acids has been proposed for increasing their lipophilicity and, consequently, to obtain multifunctional antioxidants with enhanced bioactivity and bioavailability. Hence, opportunities arise to integrate biotransformation and the extraction/fractionation of less polar derivates by a simultaneous $SCCO_2$ process. Adjusting CO_2 density by manipulating either temperature or pressure, above their critical limit (31.1 °C and 73.8 bar, respectively), allows the control of mass-transfer limitations. It is also important to consider that given the poor solubility of some antioxidants in $SCCO_2$, the addition of a polar co-solvent would be necessary. Therefore, altogether with the biocatalyst, load,

temperature, pressure, and co-solvent are considered major reaction parameters. Hence, the possibility of performing a lipase-catalyzed esterification of chlorogenic acid and 1-heptanol in $SCCO_2$, while simultaneously extracting the formed compound, has been demonstrated (Hernandez et al. 2009).

The tunable solvating power of $SCCO_2$ facilitates the separation of reactants, products, and catalysts after reaction and, hence, the integration of biocatalytic and downstream processing steps in a single bioreactor.

The synthesis of structured lipid by lipase-catalyzed trans-esterification reaction under supercritical conditions has been investigated. The reaction seems like a zero order reaction up to 6 h, and then does not proceed any more. At 323.15 K, the enzyme showed the best thermal stability. With pressure at 10.2 MPa, the enzyme had the best conversion efficiency. Due to the limitation of the substrate, the optimum molar ratio of enriched *n*-6 TAG to ω-3 PUFA was 1/4. The optimal inputs of enzyme and ethanol as co-solvent were 10 wt% of the total substrates. The reaction operating under 10.2 MPa and 323.15 K $SCCO_2$ incorporated 56 % of ω-3 fatty acids on TAG and ω-3/ω-6 = 4 at 6 h was achieved. This ratio was superior to that using hexane only reaching 18 % of ω-3 on TAG and ω-3/ω-6 = 0.7, respectively. From the results, $SCCO_2$ demonstrates a better medium for catalytic ability and stability with respect to conventional hexane solvent, including high diffusivities and low viscosities, which increase mass transfer of substrate into the catalyst particles. The activity of the enzyme maintained 81 % of initial activity after seven times of pressurization/depressurization (Lin and Chen 2008).

Fatty acid sugar esters are widely used in the food, cosmetic, detergent, and pharmaceutical industry, especially as W/O emulsifiers in food products. $SCCO_2$ is a promising alternative to conventional organic media for the lipase-catalyzed synthesis of fatty acid sugar esters. Hence, lipase-catalyzed esterification of fructose with palmitic acid was performed in 2-methyl-2-butanol at atmospheric pressure and in $SCCO_2$ with and without addition of cosolvent. The highest conversion of 78 % was obtained at 60 °C after 72 h of reaction performance at atmospheric pressure. High conversions after 24 h of reaction performance were also achieved in $SCCO_2$ at 60 °C and 10 MPa without addition of organic solvent. Reactions performed in $SCCO_2$ do not require any addition of molecular sieves. Because only palmitic acid is soluble in the supercritical phase, the separation of the fructose palmitate from remaining substrates and enzyme could easily be achieved when reaction is performed in pure $SCCO_2$. When 2-methyl-2-butanol is used as a cosolvent in $SCCO_2$, the separation becomes more difficult. However, optimization of reaction parameters for the synthesis of fructose palmitate in $SCCO_2$ without addition of organic solvent requires further studies.

12.4.5 Enzymatic Transesterification Under $SCCO_2$

One of the most commonly used reactions for modification of fats and oils is transesterification or ethanolysis in which triglycerides react with an alcohol

(such as ethanol) to produce ethyl esters of fatty acids and glycerol The unique properties of $SCCO_2$ can provide the appropriate media for transesterification in terms of mass transfer coefficient. In conventional chemical processing, the synthesis of esters by transesterification is achieved by either an acid or alkaline esterification. These catalytic reactions have low selectivity and undesirable side reactions. Moreover, the process is not ecologically friendly.

The production of fatty acid methyl ester (FAME) by direct alkali- and acid-catalyzed *in situ* transesterification of soybean flakes in CO_2-expanded methanol has been examined at various temperatures and pressures. Attempts to synthesize FAME from soy flakes via alkaline catalysis, using sodium methoxide as a catalyst, in gas-expanded methanol were unsuccessful. The authors attribute this result to the formation of carbonic acid of sodium methoxide which increases the acidity of the system and reduces the pH below what is necessary for an alkali-catalyzed transesterification to occur. However, performing the reactions in 54 mL of a 1.2 N sulfuric acid methanol mixture containing 50 % mole fraction CO_2 resulted in an 88.3 ($\pm$1.5 %) conversion of the TAG in 22.5 g soy flakes to FAME within 10 h. It was also shown that the addition of CO_2 into the system increased the rate of acid-catalyzed reactions (Wyatt and Haas 2009).

As an alternative, lipases have been used as biocatalysts for alcoholysis. Biodiesel, producible from renewable sources by transesterification, is currently of great interest as the oil price continues to increase and concerns about global warming grow. Biodiesel (fatty acid methyl ester, FAME) is an alternative fuel for diesel engines that consists of the fatty acid esters formed by the transesterification with an alcohol of vegetable oils or animal fats. Commercial biodiesel production occurs via chemical alkaline or acidic processes. Although the conventional processing of biodiesel depends on chemical synthesis to achieve a reasonable degree of conversion and reaction rates, the inherited nature of complex purification of products and energy-intensive wastewater treatment affects the sustainable production of biodiesel under a restricted environment. The enzymatic production of biodiesel can overcome these problems. Although enzymatic processes are expected to be advantageous—especially due to their reusability, better waste management, and lower energy consumption—and have been technically developed to some extent, they have not yet been industrialized because of their high enzyme cost and long reaction times. To overcome these problems, some researchers used $SCCO_2$ as a reaction medium in the enzymatic process. $SCCO_2$ offers the advantages of being non-toxic and non-flammable. As a reaction medium, it is easily separable by depressurization and the facilitated diffusion, can increase the reaction rate. Especially, this property is quite beneficial for enzymatic biodiesel synthesis, in which the reaction is often diffusionally limited due to the coexistence of immiscible phases such as oil, methanol, and solid immobilized enzyme. The resulting gaseous CO_2can then be reused.

Near critical CO_2 (NC-CO_2) has been chosen for lipase catalyzed biodiesel production due to the following two advantages over $SCCO_2$. First of all, the use of NC-CO_2 offers a more economical process due to the energy savings gained by the lower temperature condition. Secondly, oil has a higher solubility in NC-CO_2

than in $SCCO_2$, which is advantageous since the resolution of the three components (oil, methanol, and NC-CO_2) in the same phase is very important for preventing enzyme inhibition by methanol. Although the roles of $SCCO_2$ and NC-CO_2 in the mechanisms of enzymatic biodiesel synthesis are not clear, one possible idea is that they improve the solubility of methanol in the reaction mixture, preventing the enzyme from encountering high methanol concentration environments because of their somewhat polar nature (Lee et al. 2012). Additionally, Lipozyme TL IM showed to be the most efficient biocatalyst for biodiesel production under NC-CO_2 (Lee et al. 2013a). It is well known that excess amounts of methanol inhibit lipase activity during enzymatic FAME synthesis. To overcome this problem, the stepwise addition of methanol is generally adopted (Shimada et al. 1999). Moreover, a by-product of the biodiesel production, glycerol, can also inhibit the enzyme or cause fouling of the immobilized enzyme. The removal of glycerol, a potential inhibitor, as part of each batch's processing, has to be considered in the bioprocess (Dossat et al. 1999).

Enzymatic biodiesel approach showed promising results due to their high selectivity and mild operative conditions. Enzymatic transesterification reaction is similar to conventional transesterification, except that they are catalyzed by a variety of biological catalysts rather than chemical catalysts. In contrast to conventional processes, biocatalysts can transesterify TAG with high free fatty acid content. In Table 12.2 some of the most relevant studies of transesterification reaction catalyzed by lipases in $SCCO_2$ are shown.

Table 12.2 Summary of studies of transesterification reaction catalyzed by lipases in $SCCO_2$

	Ref.
Supercritical fluid assisted, integrated process for the synthesis and separation of different lipid derivatives	Weber et al. (2008)
Lipase-catalyzed alcoholysis with supercritical carbon dioxide extraction 1: Influence of flow rate	Gunnlaugsdottir and Sivik (1997)
Continuous lipase-catalyzed production of fatty acid ethyl esters from soybean oil in compressed fluids	Dalla Rosa et al. (2009)
Enzymatic alcoholysis of palm kernel oil in n-hexane and $SCCO_2$	Oliveira (2001)
Fatty acid ethyl esters production using a non-commercial lipase in pressurized propane medium	Hildebrand et al. (2009)
Enzymatic conversion of corn oil into biodiesel in a batch supercritical carbon dioxide reactor and kinetic modeling	Ciftci (2013)
Continuous production of fatty acid methyl esters from corn oil in a supercritical carbon dioxide bioreactor	Ciftci (2011)
A review of enzymatic transesterification of microalgal oil-based biodiesel using supercritical technology	Taher et al. (2011)
Economic analysis of a plant for biodiesel production from waste cooking oil via enzymatic transesterification using supercritical carbon dioxide	Lisboa et al. (2014)
Enzymatic biodiesel synthesis in semi-pilot continuous process in near-critical carbon dioxide	Lee et al. (2013a)

(continued)

Table 12.2 (continued)

	Ref.
Optimization of enzymatic biodiesel synthesis using RSM in high pressure carbon dioxide and its scale up	Lee et al. (2013b)
Supercritical synthesis of biodiesel	Bernal et al. (2012)
Immobilised lipase on structured supports containing covalently attached ionic liquids for the continuous synthesis of biodiesel in $SCCO_2$	Lozano et al. (2012)
Characteristics of menhaden oil ethanolysis by immobilized lipase in supercritical carbon dioxide	Shin et al. (2012)
Continuous production of biodiesel from fat extracted from lamb meat in supercritical CO_2 media	Al-Zuhair et al. (2012)
Improved high-pressure enzymatic biodiesel batch synthesis in near-critical carbon dioxide	Lee et al. (2012)
Continuous enzymatic production of biodiesel from virgin and waste sunflower oil in supercritical carbon dioxide	Rodrigues et al. (2011)
Biodiesel production by a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases using a supercritical carbon dioxide process	Lee et al. (2011)
Synthesis of biodiesel in supercritical alcohols and supercritical carbon dioxide	Varma et al. (2010)
Biodiesel production from various oils under supercritical fluid conditions by *Candida antarctica* Lipase B using a stepwise reaction method	Lee et al. (2009)
Synthesis of biodiesel from edible and non-edible oils in supercritical alcohols and enzymatic synthesis in supercritical carbon dioxide	Rathore and Madras (2007)

Concluding Remarks

Supercritical fluids have demonstrated to be an environmentally friendly media for extraction, chemical and enzymatic reactions and related processes in the field of fat, lipid and derivative products. Many new processes and products have been, are being, and will be developed based on this technology, by using the intrinsic physical and chemical properties of supercritical fluids.

Combination of different methodologies under a high pressure environment offers new possibilities to supercritical technology for obtaining faster and more efficient extractions, new extracts without thermal degradation and adjustable selectivity.

Finally, the scenario for exploitation of biocatalytic reactions in CO_2 media with the view to develop new biotransformation schemes for novel lipid-type products manufacture seems to be highly promising. Besides the benefits and flexibility of supercritical and near-critical solvent systems in bioprocessing, it is advantageous and in many cases necessary, to carry out additional processing operations either before or during supercritical fluid

(continued)

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processing and to consider it as a whole integrated process. This approach will achieve better overall fractionation, bioconversions of specific lipid compounds and utilization of the supercritical fluid solvent state to enhance or create new processing opportunities that could not be realized in conventional solvent systems.

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Chapter 13
Development of Multiple Unit-Fluid Processes and Bio-refineries Using Critical Fluids

Jerry W. King and Keerthi Srinivas

13.1 Introduction

Supercritical fluids and their liquefied analogues have been traditionally used in single unit operations, i.e. extraction, fractionation, using neat $SCCO_2$ or with appropriate modifiers (McHugh and Krukonis 1994). Since the 1980s, almost 38 % of the supercritical fluid extraction processes have been devoted to extraction of food and natural products (Valcarcel and Tena 1997). Beginning in the mid-1980s, columnar and chromatographic techniques followed by reactions in supercritical fluids were developed to facilitate supercritical fluid derived extracts or products (King 2004a, b), thereby extending the application of a critical fluids processing platform beyond SFE. These newer developments were investigated in part due to the complexity of many natural product matrices and the desire to concentrate specific target components for food and other industrial uses, as illustrated in Fig. 13.1.

We have found that the generic solvation properties of the two principal critical fluids, CO_2 and water, to be explained by an extended solubility parameter (δ) approach (King et al. 2006, 2007). Hence, by adjustment of pressure and temperature for CO_2, or temperature in the case of water, one can optimize the solubility of solutes or reactants in these media, or predict their miscibility, by comparing their relative solubility parameters as a function of temperature and pressure. Such an approach has a practical value considering the molecular complexity of many solute types processed in critical fluids. Their solubility parameters or solute-fluid

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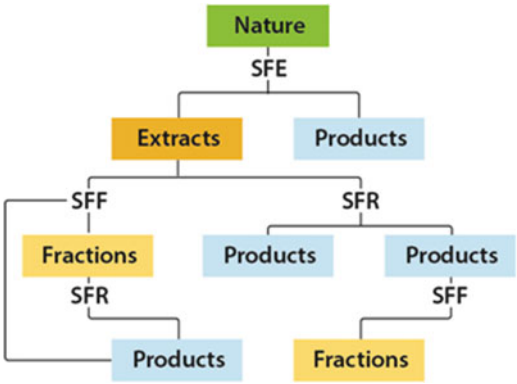

Fig. 13.1 A generic scheme for processing materials using combinations of supercritical fluid extraction (SFE), supercritical fluid fractionation (SFF), and supercritical fluid reactions (SFR)

interactions can be explained by using the Hansen three-dimensional solubility concept which allows the application of functional group contribution methods for calculating requisite physical property data as well as solute or solvent solubility parameters as recently reported by Srinivas et al. (2008). As indicated in Fig. 13.2, manipulation of the δ of CO_2 is the basis of the unit operation of supercritical fluid extraction (SFE) which through compression of the CO_2 allows some degree of selective extraction of targeted solutes to be achieved, and upon CO_2 decompression, fractionation of the extracted components via integrated separator vessels.

Similarly, the reduction in water's total solubility parameter with increasing temperature is largely due to a reduction in the hydrogen-bonding propensity as reflected by its hydrogen-bonding solubility parameter component (Panayiotou 1997). Water does not attain the solvation properties of solvents like ethanol or methanol until quite elevated temperatures which is in contrast to the often cited dielectric constant concept which is invoked to explain the solvent properties of subcritical water (Hawthorne et al. 1994). The solvent properties of water as described by the solubility parameter concept has some implications with regard to its use as a "green" solvent and a substitute for ethanol in hydroethanolic-based extractions which are GRAS (Generally Regarded as Safe)-approved food processing solvents. Its substitution for ethanol as a processing medium is highly desired to save on processing costs, its separation from water in solvent recycle schemes, and oversight by revenue authorities. These are some of the factors which accelerate research in the use of subcritical water for the extraction of natural products and nutraceutical food components. This is part of the overall critical fluid technology platform using only water and carbon dioxide which result in widespread utilization (King 2000a, b).

Traditional oleochemical processing operations such as fat splitting or hydrogenation are often conducted under either subcritical or supercritical processes. Fat-splitting processes such as Twitchell process (Lascaray 1949) or Colgate-Emery synthesis (Barneby and Brown 1948) utilize temperatures and pressures in excess of the boiling point of water under the appropriate pressure, but below the critical point of water to facilitate the hydrolysis of triglycerides to fatty acids.

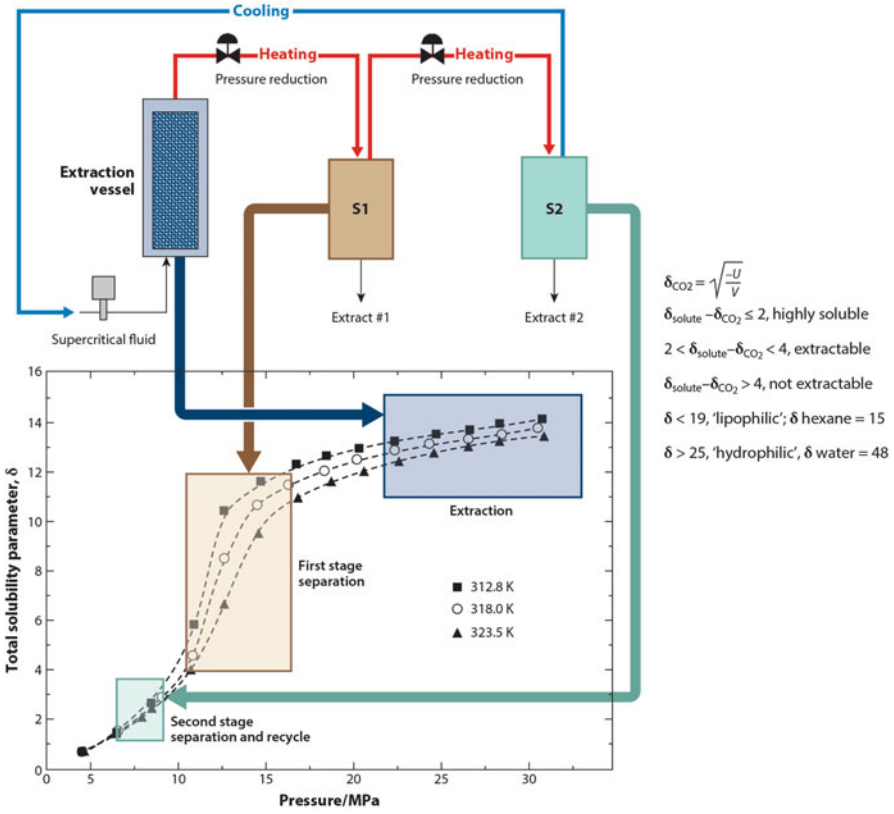

Fig. 13.2 Variation in the total solubility parameter of CO_2 as a function of pressure and temperature correlated with its use in the unit process of SFE and SFF using selective depressurization to separate constituents in the extract (King 2014)

However, it should be noted that these processes were frequently interpreted as steam-based hydrolysis rather than hydrolysis using sub-critical water. Hydrogenations using binary mixtures of CO_2-H_2 or propane-H_2 are supercritical with respect to the pure component critical constants, but reaction conditions are conducted under less dense conditions due to the high temperatures involved, hence the cited rapid kinetics associated with hydrogenations conducted under these conditions (King et al. 2001) are due to accelerated mass transfer effects as opposed to reactant solubility enhancement.

The advantages of coupling processing options using critical fluids have been discussed by King and Srinivas (2009). Several specific options are illustrated for the case of processing essential oils (King 2003) using pressurized fluids. Six discrete unit processes were noted which included traditional SFE with $SCCO_2$, SFF employing stage-wise pressure reduction (like Fig. 13.2), SFF using columnar-based deterpenation (Reverchon 1997), supercritical fluid chromatography (SFC), another variant of SFF called subcritical water deterpenation (Clifford et al 1999),

and utilization of a $SCCO_2$ or LCO_2 with a permselective membrane described by Towsley et al. (1999).

13.2 Multiple Critical Fluid Processing Platforms

In the broadest sense, multiple critical fluid processing involves the integration of two or more fluids held under pressure applied as either mixtures or in a sequential manner for one or more unit processes. Solubility of solutes and reactants in $SCCO_2$ has been extensively studied and a recent tome has assembled much of the available data (Gupta and Shim 2007). Likewise there is a fair understanding as to the choice of a suitable co-solvent to pair with $SCCO_2$ to enhance the solubility of more polar solutes in the compressed CO_2 medium, although binary phase equilibria data is not always available over the desired range of pressure–temperature for such systems. This is critical if one is concerned with operating in the one phase supercritical fluid region with respect to both components, however as noted by several investigators (Bogel-Lukasik et al. 2008; Licence et al. 2004; Eckert et al. 2007), there are several examples where processing can be done with $SCCO_2$-co-solvent systems in the two phase region. This situation becomes of interest particularly when large amounts of an organic co-solvent are used in conjunction with $SCCO_2$ to enhance the solubilization of a solute which exhibits limited solubility in neat $SCCO_2$. The critical question then becomes whether another compressed fluid might better be integrated into the design of the process.

As remarked previously, compressed water at high temperatures and pressures, i.e., supercritical water has been extensively investigated for many years. In the 1990s a similar but somewhat more diffuse focus on using water in its subcritical state with respect to its critical temperature (T_c) received attention due to its application as a reaction medium to transform organic chemicals and biomass into targeted products (Antal et al. 2000; Savage et al. 1995). Concurrently, particularly in the field of analytical chemistry, subcritical water and other subcritical fluids were explored as alternative extraction solvents under external compression above their boiling points (King 2004a, b; Yang et al. 1995; Ayala and de Castro 2001). Analytical methods developed with the use of pressurized solvents essentially use subcritical fluids above their boiling point—the pressure applied frequently is far in excess of what is required by inspection of the V-L (vapor-liquid) curves for these fluids (King 2006). Unfortunately, researchers in these disparate areas despite using a common compressed fluid, water, have not always recognized the generic utility of water as a universal compressed fluid medium as well as "green" complimentary solvent to compressed CO_2 (King et al. 2006).

Using the Hansen three-dimensional solubility parameter approach coupled with SPHERE and Hsp3D (Hansen 2007) software programs, we have studied the interaction between subcritical water and complex organic solutes, including biopolymers, as a function of temperature. Water under adequate compression, has the ability by adjustment of the applied temperature and pressure, to serve as an

extraction solvent as well as a reaction medium depending on what unit operation is desired. Residence time of the solute (reactant) in the aqueous medium thus becomes a critical parameter in conducting extractions above the boiling point of water and for optimizing reaction conditions "higher up" the V-L curve for water. There appears in our opinion the lack of rationale design for choosing reaction conditions in sub-critical water, although the semi-empirical "severity" parameter often-cited in biomass conversion studies is one attempt to quantify the required hydrolytic conditions (Galbe and Zacchi 2007). Using the solubility parameter approach, we have shown that conditions which correspond to the solubilization of the carbohydrate oligomers, i.e., hemicellulose and cellulose, are the optimal conditions for depolymerizing these carbohydrate polymers (King and Srinivas 2009). Similarly, we have also used this approach for other biopolymers pretreated in subcritical water such as chitin and rationalized the difficulty in dissolving lignin-type polymers in subcritical water (King et al. 2006).

Recently, compressed gases in their supercritical fluid state, particularly $SCCO_2$, dissolved in liquids and subcritical liquids have become of interest as *in-situ* catalysts or modifiers for reaction and extraction unit processing. The use of $SCCO_2$ as a replacement for metallic catalysts in glycerolysis reactions was reported by Temelli et al (1996) and a review of its use in synthetic organic reaction chemistry has been published by Rayner et al. (2005). Dissolving $SCCO_2$ under pressure in pressurized water creates a versatile medium with respect to acidic-based extraction chemistry and reactions due to the inherent carbonic acid equilibrium that is pressure dependent as studied by Toews et al. (1995). We and others have found that if sufficient CO_2 under pressure is applied to aqueous solutions, that pH's between 2.0 and 2.5 can be achieved. The basis of this low pH is due to enhanced dissolution of CO_2 as its pressure is increased in aqueous solution (Teng and Yamasaki 1998; Weibe and Gaddy 1934; Sabirzyanov et al. 2002; Stewart and Munjal 1970). Intuitively, increasing the temperature of water should decrease the amount of gas dissolved in water at lower pressures and temperatures, however as more pressure is applied to $SCCO_2$, the amount of dissolved gas in water increases lowering the solution pH. This control of solution pH by dissolution of $SCCO_2$ can also affect the equilibrium-between pH-sensitive solutes that can be extracted using subcritical water, i.e., anthocyanins and similar flavonoid-based solutes (Clifford 2000). This technique offers definite advantages with respect to avoiding the use of mineral acids in extraction and reaction chemistry since the dissolved $SCCO_2$ can be jettisoned to the atmosphere or recycled by a reduction in pressure. Studies using supercritical carbon dioxide as reaction solvent especially in catalytic hydrolysis as described above have also shown good product separation characteristics by increasing the pressure from 2 MPa to as high as 12 MPa. Approximately 90 % of the hexanes were successfully separated from the hydrolytic mixture dissolved in supercritical carbon dioxide (Rayner et al. 2006).

13.3 Multiple Units Processing: Concepts and Possibilities

The coupling of SFE with more traditional methods for processing naturally-derived plant oils is one example of coupling unit processes. The concept of continuous processing of oils from seeds and meals goes back to the mid-1980s with the description of the operation of an Auger-type screw press by Eggers (1996). Here, a supercritical fluid such as $SCCO_2$ is used to assist in the removal of oil from crushed seeds or meals which may have been partially pre-extracted. The physicochemical basis of the process is still not well understood, but involves the addition of liquefied CO_2 to the seeds or meal inside an expeller barrel to aid in the oil extraction process. The hydraulic compression on the seed meal creates considerable pressure and heat on seed matrix resulting in the conversion of the added CO_2 to its supercritical state. The hot compressed carbon dioxide partially solvates the seed oil akin to what occurs when SFE is performed with $SCCO_2$, but more importantly dilutes or expands the expressed oil enhancing its removal from the seed or meal bed. Methods to achieve this goal have been described in the patent literature most notably by Foidl (1999) whose process has been partially commercialized and applied to the processing of soybeans.

This CO_2-assisted expression process has been commercialized by Crown Iron Works in Minneapolis, Minnesota under the trademark of HIPLEX process and CO_2 expression demonstrated on a Harburg Freudenberger expeller having a 25 ton per day capacity. Such a process is in commercial operation at SafeSoy Technologies in Ellsworth, Iowa as show in Fig. 13.3 below. The ratio of oil to CO_2 is 3:1 which reduces the vegetable oil viscosity by 1/10 resulting in between (80–90) % vegetable oil recovery for soybeans and over 90 % recovery of canola oil. Such solvent-free oils and meals are superior in quality to solvent extracted products.

A similar approach would be welcomed for subcritical water extraction of natural and food-related products as well as for the conversion of biomass substrates on a continuous basis in which the substrate to be extracted or treated with pressurized water would be contacting as a slurry with the pressurized water. This could then be applied to such diverse matrices as grape pomace, cocoa beans, and herbal substances provided the residence times in the expeller are minimized. Current systems for affecting such pressurized water extractions are staged as semi-continuous batch systems or by combining the substrate to be processed as aqueous slurry with water before passage through a heated extraction vessel. It should be noted that critical fluid-based expeller processes compete with similar unit processing done with the aid of extruders (Giezen et al. 2005). Although extruders have shown promise in the processing of finished food products (Rizvi et al. 1995) their attendant expense and lower throughputs make them less attractive than the expeller-based processes described above.

Integrating critical fluid technology with membranes has permitted the separation of low and high molecular weight compounds obtained from the $SCCO_2$ extraction of lipids from foodstuffs such as butter or fish oil using nano-filtration membranes (Sarrade et al. 1999). Similarly, it has been reported that is possible to

Fig. 13.3 Continuous SFE coupled with an oil seed expeller to produce defatted meals

extract polyphenols from cocoa seeds using neat $SCCO_2$ and with ethanol as a co-solvent, and then concentrating the extract using polymeric nano-filtration or reverse osmosis membranes. This system operated at a pilot scale between (8 and 15) MPa and at 40 °C resulted in a maximum yield of polyphenols of 43 % when the pressure was optimized at 8 MPa using ethanol. The study also indicated a high performance of all the membranes when the trans-membrane pressure was maintained in excess of 1 MPa (Sarmento et al. 2008). The ability to concentrate extracted polyphenols using $SCCO_2$ extraction paired with membranes suggest that a similar tandem involving subcritical water–membrane coupling would be advantageous since extraction with subcritical water results in a diluted extract. This concept was first advanced by King (2002) and noted in a US patent issued to Wai and Lang (2003). They suggested that SFE could be implemented on a natural product matrix followed by subcritical water extraction sequentially on the same matrix and then followed by a membrane separator to yield a concentrate of the aqueous extract.

On a laboratory scale, a SFR-SFR coupling has been used to synthesize mixtures of fatty alcohols. The generation of fatty acid methyl esters (FAMES) in this case was based on studies involving the enzymatic synthesis of FAMES directly from vegetable oils dissolved in $SCCO_2$ (Jackson and King 1996; Snyder et al. 1997). Combining this transesterification reaction with a hydrogenation reaction using consecutively coupled packed bed reactors allowed the production of FAMES in either $SCCO_2$ or SC-C_3H_8 followed by exhaustive hydrogenation of the FAMES to fatty alcohols. In this process, a non-chromium-based catalyst was used to

successfully convert the FAMES derived from soybean oil to a mixture of $C_{16}+C_{18}$-saturated alcohols at 250 °C and 25 mol% H_2 in $SCCO_2$. This is an excellent example of how a two-step synthesis process can be conducted in supercritical fluid media that is also environmentally-benign by permitting reuse of the critical fluid media as well as the reaction by-product from the hydrogenation step (methanol).

It is impossible to separate in the multiple unit and fluid processing platform the role of fluid interchange with tandem unit processing. Toward this end, the choice of solute or substrate modification and/or fluid medium can enhance the opportunity to utilize the various combinations of fluids or unit processes. Two will be cited here: (1) formation of methyl esters of lipid-type solutes such as fatty acids (FAMES), and (2) use of water primary for hydrolysis of complex naturally-occurring substrates. FAMES are an extremely versatile modification for the critical fluid processing of fats/oils and their oleochemical derivatives. Aside from the formation of FAMES for conversion to biodiesel via enzymatic synthesis (Gupta et al. 2004) or in sub- and super-critical methanol (Bunyakiat et al. 2006), FAMES or similar esters can be used advantageously in SFE (Quancheng et al. 2004), columnar modes of SFF (Eller et al. 2008), supercritical fluid chromatography (SFC) (Pettinello et al. 2000), and as noted above in a SFR sequence. This versatility is due to one or more of the following factors relative to the non-methylated analogs: (1) enhancement of solute volatility or solubility, (2) improvement of separation factor (α), or (3) intermediate formation for downstream synthesis, and analytically-useful derivatives. Formation of FAMES before utilizing multi-unit processing can allow easier SFF of fatty acids (Brunner 2000), selective SFE and SFR of fatty acids from tall oil (Taylor and King 2001) for subsequent conversion to biodiesel, and to fractionate soapstock (King et al. 1998) or deodorizer distillate (Nagesha et al. 2003). For example, countercurrent multistage processing of edible oils using critical fluids has been shown to be capable of producing fatty acid esters, tocopherols, squalene concentrate, sterols, and fractionated triglyceride mixtures. Approximately 70 % of the fatty acid methyl esters in deodorizer distillates plus tocopherols and sterols can be extracted with $SCCO_2$ (Fang et al. 2007). Tocopherols and sterols in the resultant extract can then be separated from the FAMES by columnar SFF via countercurrent fractionation using $SCCO_2$.

13.4 Examples of Integrated Critical Fluid Processing

The above typical results of employing CO_2 extraction in tandem with pressurized liquid fluids suggest an interesting option and current trend in employing this mixed pressurized fluid matrix as both extraction and reaction media. As noted previously, the incorporation of pressurized CO_2 into subcritical water, i.e., a gas-expanded liquid, makes for an interesting extraction and reaction medium. The Meireles group in Brazil (Moreschi et al. 2004; Pasquel et al. 2000) have utilized this principle in the processing of ginger bagasse both as a pre-treatment step and to

degrade bagasse to sugars for potential fermentation. Pretreatment with $SCCO_2$ seemed to yield somewhat ambiguous results since the authors state that non-treated bagasse was hydrolyzed more effectively then CO_2-pretreated bagasse (Moreschi et al. 2006); the latter process was hypothesized to degrade the oleoresinous materials in the bagasse matrix. Their results for matrix pretreatment with $SCCO_2$ are in stark contrast with other reports in the literature that indicate $SCCO_2$ pretreatment is an effective procedure proceeding biomass degradation (Kim and Hong 2001). It should be noted that the above results are somewhat different than using the previously-mentioned carbonated water to hydrolyze carbohydrate polymers; also $SCCO_2$ is effective in removing high value components from the biomass matrix prior to hydrolyzing the biomass matrix (Moreschi et al. 2006). Since reported $SCCO_2$ pretreatment methods exist and carbonated water hydrolysis has been shown by us (King et al. 2008) and others (Van Walsum and Shi 2004) to be an effective hydrolysis medium, the above ambiguity may be due to the varying recalcitrance of the target biomass matrix to hydrolytic degradation. The hydrolytic action patterns of carbonated water can vary quite significantly depending on the matrix being hydrolyzed although the hydrolysis temperature and residence time can be varied to produce optimal depolymerization of the constituent carbohydrate polymers inherent in the biomass.

One aspect of our current research focuses on the application of critical fluids for processing grapes and grape by-products and similar natural antioxidant-containing matrices. These matrices and target solutes are a fruitful area in which to apply combinations of mixed critical fluid and unit processing steps. One of the seminal questions is whether $SCCO_2$ and co solvent combinations or a hot pressurized fluid such as water or ethanol—or combinations thereof—are most appropriate for extracting and fractionating the targeted solutes. There is a considerable literature in the application of $SCCO_2$ for extracting grapeseed oil (Gomez et al. 1996; Cao and Ito 2003) as well as further fractionating the extract to enrich certain polyphenolic constituents. Recovery of solutes such as gallic acid, catechin, epicatechin, etc. via a $SCCO_2$-based method almost always require the use of methanol or ethanol as co solvents (Murga et al. 2000).

Other studies have utilized subcritical water to extract procyanidin compounds and catechins from grape processing wastes (Garcia-Marino et al. 2006). Extractions conducted at approximately 10 MPa and in the temperature range of (50–150) °C were adequate to recover and fractionate gallic acid, procyanidin dimers, and the corresponding oligomers from the grape pomace using an analytical scale pressurized fluid extractor. Aside from water and hydroethanolic pressurized fluid extraction, sulfurized water has also proven effective for the extraction of anthocyanins and procyanidins from grape pomace (Ju and Howard 2005). This parallels similar work by the senior author in using neat and acidified water to extract anthocyanins from berry substrates using both ASE and a batch continuous subcritical water extractor. As noted in the patent issuance on this process (King and Grabiel 2007), residence time of the extracted solute in the hot pressurized water must be minimized to prevent degradation of the anthocyanin moieties or their possible reaction with sugars to other products. It is unknown at this time whether such side reactions

in pressurized water could be generating antioxidant moieties, but the potential ability to control the ratio of polyphenolic stereoisomers and to depolymerize or repolymerize biologically-active antioxidant oligomers in pressurized fluid media could be a significant area for future research—particularly if they come from cheap and renewable natural resources (King et al. 2007).

Another example of integrated unit processing based on the use of $SCCO_2$ is the aforementioned glycerolysis of vegetable oil feedstocks (Temelli et al. 1996) to produce industrially useful mixtures of mono-, di- and tri-glycerides. As noted in Fig. 13.4, this can be the initial unit process in a SFR-SFF coupling in which the produced glyceride mixture is then fed from the stirred reactor into the bottom section of a packed fractionation column. The glyceride mixture is then further fractionated by superimposing a thermal gradient over the four sectors of the fractionating column noted on the right-side in Fig. 13.4, resulting in a product at the top of the column enriched in mono-glyceride content (>90 wt%) while the raffinate fraction at the bottom of the column contains the higher molecular weight glycerides. Such a scheme makes dual use of the $SCCO_2$ obtained from a central compressor source thereby saving the energy required by the integrated process. The CO_2 that separates from enriched mono-glyceride fraction at the top of the fractionating column can then also be recycled to either the bottom of fractionating column and be reused, or alternatively be used to feed the glycerolysis reactor on the left-side in Fig. 13.4.

Kusdiana and Saka (2004) has demonstrated a two-step process for the production of biodiesel based on the Saka—supercritical methanol process for converting both fats/oils and free fatty acids to biodiesel. A coupled process called the Saka-Dadan process however, uses subcritical water in front of the above Saka process for the hydrolysis of fats/oils to free fatty acids followed by supercritical methanolysis of the resultant free fatty acids to FAMES. A pilot scale unit of this process is in operation in Fuji City, Japan. This overall biodiesel production platform is an excellent example of a critical fluid-based SFR-SFR integrated process.

Similarly, Baig et al. (2008, 2013) have recently reported on the combined critical fluid treatment of sunflower oil to yield value-added substances as well as a model for the "critical fluid bio-refinery". This concept can be achieved by coupling two or more reaction processes into one continuous flow system; namely the subcritical water hydrolysis of sunflower oil triglycerides to free fatty acids followed by esterification of the free fatty acids to FAMES in $SCCO_2$ using lipase catalysis. The subcritical water extractor conditions were maintained at (250–390) °C with pressures as high as (10–20) MPa using oil: water ratios of 50:50 and 80:20 (v/v). The supercritical fluid enzymatic-based esterification process was operated at temperatures (40–60) °C using a Novozyme enzyme catalyst. The subcritical water results indicated a high rate of conversion at higher temperatures (330 °C) followed by possible degradation of the free fatty acids when exposed to longer residence times. A yield of approximately 90 % hydrolyzed free fatty acids was achieved in 25 min at 330 °C or for 45 min at 310 °C. The esterification process yielded between (60–70) % FAMES at a pressure of 20 MPa and 60 °C with low enzyme concentrations.

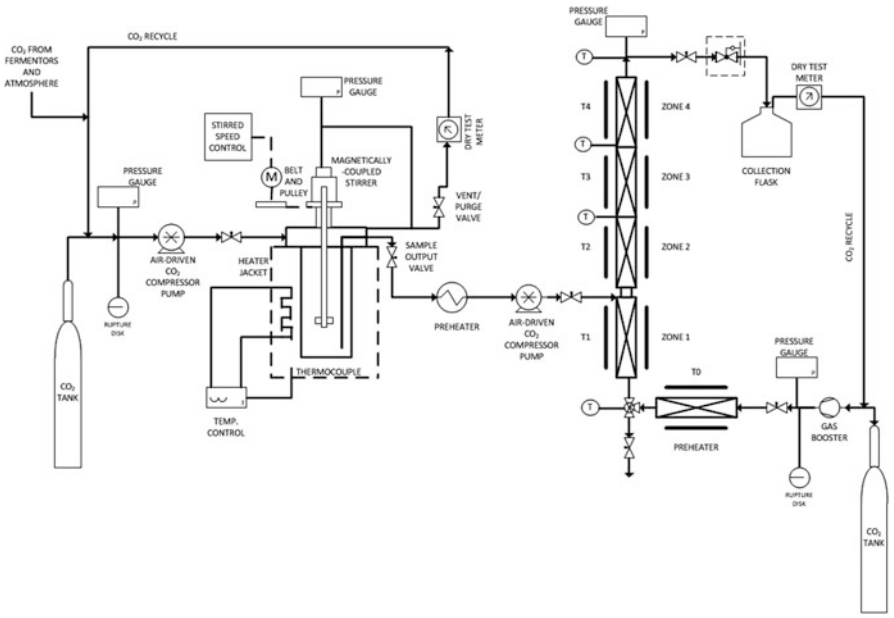

Fig. 13.4 Coupling of a $SCCO_2$-catalyzed reaction (SFR) for the production of a mixed glyceride product transferred to a columnar fractionation unit (SFF) for further enrichment of glyceride fractions

13.5 The Multi-fluid Plant Concept

As is evident in the prior discussion, sub- and $SCCO_2$ are the most prevalently used fluids for SFE and related processes. Hence, it is unlikely that most pilot and industrial scale plants will be designed for use with other fluids having widely different critical temperatures or pressures. One exception is the documented use of sub- and supercritical propane, particularly as used for defatting of food related substrates or extraction of high value oils, such as fish oils. The extraction-fractionation schemes using C_3H_8 are commonly between (80–120) °C and corresponding requisite pressures (which are much lower than those required for $SCCO_2$ extraction). Such conditions allow propane to outperform $SCCO_2$ in terms of its ability to solubilize more oil and lipid constituents on a weight percent basis; however the downside of propane use is its flammability and hydrocarbon-like solvent properties.

Over the past 10 years, subcritical water slightly above the boiling point of water but under very modest compression (<2 MPa) has seen increasing use and advocacy in the processing of foodstuffs and botanicals. This regime of subcritical water must be distinguished from supercritical water used at high temperatures which is virtually destructive to all organic matter, and it must also be noted that temperature

and pressure range (100–160 °C, 0.2–2.5 MPa) normally applied is far less than the subcritical water conditions used in biomass liquefaction to fermentable sugars. Hence a plant designed to perform subcritical water extraction-fractionation processes can be built for substantially less cost than a plant employing $SCCO_2$.

So why is there interest in using subcritical water for extracting botanicals and biomass waste streams? They are as follows:

- Water complements $SCCO_2$ and liquid CO_2 (LCO_2) as a "green", GRAS, and consumer- and environmentally-benign solvent
- By adjusting the extraction temperature and modest pressurization and optimizing the fluid velocity through the extraction bed, subcritical water can be used as a replacement for ethanol or hydroethanolic mixtures as solvents
- Reduction in the extraction temperature makes subcritical water a more non-polar solvent, in fact and excellent solvent for polyphenolic solutes which constitute many of the natural antioxidant mixtures that are sold commercially. Total extraction of these natural antioxidant mixtures is difficult and compromised when attempting their removal via $SCCO_2$ with and without organic co-solvent addition.

There are hidden benefits and factors which derive from the billeted rationale provided above. For one, botanical and herbal extractors would like to terminate or minimize the use of ethanol in their extraction protocols. The rational for this is partially process economics, but also the alleviation from having to keep a rigorous mass balance of ethanol use for the tax authorities—at least in the USA. Low pressure subcritical water extraction can also be utilized on the "front end" of a biomass pretreatment process to remove high value botanicals before conversion of the biomass matrix via depolymerization to sugars or pyrolysis to a liquid oil, or gasification. Therefore the high value extractives can be removed from the biomass using subcritical water, before the same media is used to process the remaining biomass for fuel or chemical use (Srinivas and King 2010).

So what are the implications in terms of designing a plant for multi-fluid high pressure operation, namely that the same plant equipment used for $SCCO_2$ SFE can also be used for subcritical water processing—this includes the pumps, vessels, etc. Hence one can achieve an integrated solvent platform that can extract all of the bio-actives present in the botanical or marine matrix. Subcritical water processing, unlike $SCCO_2$ does not require the removal of water from biomass matrix, and can be a powerful approach particularly if coupled with a downstream membrane concentration process. This is not to be confused with the interesting research pioneered by Sims in the late 1980s in which a membrane separator was coupled to a flowing stream of $SCCO_2$ or LCO_2. Conceptually, one can take the biomass source and process it first with $SCCO_2$ followed by subcritical water extraction (SWE). The converse process is also possible, water before CO_2—either way extract fractions of discrete molecular composition are facilitated. A common bridge co-solvent for these two media—if necessary—would be ethanol because of its GRAS status, and the fact that it is a bio-renewable solvent. The carbonation

of subcritical water at high CO_2 pressures as noted previously also introduces some unique extraction selectivity as well as affecting solute chemical equilibria that can be exploited for pH-sensitive solutes. Subcritical water as a processing medium can be applied in a columnar countercurrent mode to fractionate extracted chemicals, such as in the deterpenation of essential oils as demonstrated by Clifford et al. (1999).

One final important point to emphasize, $SCCO_2$ extraction using high pressures and/or accompanied by the use of a co-solvent permitting extraction of more polar moieties from the biomass than can be achieved by neat $SCCO_2$ alone—has definite limitations. If one considers the aforementioned extraction of berry or grape pomace, $SCCO_2$ employing a co-solvent at even higher pressures will not extract all of the available bioactive solutes—or require copious amounts of co-solvent and long extraction times—to achieve total recovery of the available bioactive material. A better strategy would be to extract the grape or berry seed oil with $SCCO_2$ followed by subcritical water extraction of the polar solutes, i.e. the polyphenolic-containing fraction. Slowly several companies that produce equipment for supercritical fluid-based processing have begun to design systems for dual fluid use by offering laboratory and pilot plant prototypes.

Another two-fluid option for processing consideration is the use of dimethyl ether (DME) in tandem with CO_2. For example, CO_2 can be used initially to process typical non-polar lipid moieties, followed by the utilization of DME to process polar lipids. The merits of using neat DME and in conjunction with CO_2, have been described in a review by Catchpole et al (2012). Of particular interest is the high solubility for polar lipids, such as soybean phospholipids in DME (~20 wt%), as well as in DME + water mixtures, using water as a co-solvent (~30 wt%). This is due to the fact that DME-water mixtures under compression have a higher δ relative to $SCCO_2$ which overlaps with the calculated δs for phospholipids, such as phosphatidyl choline (18.3 $MPa^{1/2}$), phosphatidic acid (18.8 $MPa^{1/2}$), phosphatidylethanol amine (19.1 $MPa^{1/2}$), and phosphatidyl inositol (22.3 $MPa^{1/2}$). A continuous process for extraction of egg yolks and aqueous whey protein concentrate streams are described by Catchpole et al. (2006) in which a liquid feed stream is contacted continuously with DME through an in-line static mixer. Lipid extraction ranged from (60 to $\geq$ 90) % recovery depending on the feed material and processing conditions. Although DME has some of the same disadvantages exhibited by propane, namely high flammability, this can be partly suppressed by using mixtures of DME and CO_2. The ability of DME (which can be used in its subcritical state) and its mixtures with water to extract wet biomass such as algae, dairy products, and fermentation-derived solutes—such as natural pigments—at lower pressures relative to those required for $SCCO_2$ argues for its use in the processing of foodstuffs, fermentation media, and natural products.

13.6 Critical Fluid Bio-refineries

The authors have previously noted the considerable potential for applying critical fluids in bio-refineries, etc. (King et al. 2006) but several additional examples are worth citing. Often times, a bio-refinery is compared with an existing industrial process such as a petroleum refinery (Kamm et al. 2006). Bio-refineries employing critical fluid media maybe based on the material or commodity being processed—such as an agricultural commodity, biomass, or a renewable, suitable fuel source. Whereas all possibilities cannot be covered in this chapter, the following critical fluid-based are discussed:

- A palm oil refinery
- A canola oil refinery
- A rice processing refinery
- $SCCO_2$ algae-based bio-refinery
- Biogas production refinery
- Total hydrolysis refinery

Because of the relative high solubility of lipid-based materials in $SCCO_2$, several vegetable oil-based bio-refineries have been described in the literature. The Brunner group (Chuang and Brunner 2006) at the Technische Universitat Hamburg-Harburg (TUHH) has used a combination of multi-stage SFE accompanying by selective adsorption-desorption on an *in-situ* adsorbent followed by final enrichment of the target tocopherol and carotenoid components to obtain highly pure isomers of the major tocopherols and tocotrienols contained in palm oil. Cold pressed palm oil (CPO) is vacuum distilled resulting in a deodorizer distillate allowing greater rectification to be achieved then could be accomplished using just the CPO. Thus tocopherols contained in the CPO between (500–1,000) ppm concentration levels can be enriched along with the carotenoids to (20–50) wt. % concentrates. Using more selective SFE conditions along with an adsorbent, these concentrates can be further concentrated to between (70–100) wt. %. The final stage of these coupled processes, SFC results in 95 wt% pure alpha-tocopherol isomer which is the precursor to Vitamin E.

Temelli (2009) has hypothesized an integrated biorefinery based on such feedstocks as canola oil. Here the lipid rich biomass is selectively extracted via SFE with $SCCO_2$ leaving behind a protein-carbohydrate rich residue. The $SCCO_2$-lipid extract can then be further fractionated using a packed fractionating column to enrich the top extract with a potential nutraceutical ingredient while the raffinate oil can be further treated in a SFR sequence to produce a specific oleo-based chemical or biofuel. Recently, this latter option has been applied to corn distiller grains (DDGS) by Ciftci and Temelli (2013) to convert the $SCCO_2$-lipid extract to biodiesel (FAMES) using a packed bed reactor. SFE conditions were optimized between (35–50) MPa and (40–70) °C to enhance the concentration of nutraceutical

bioactives in the extract. FAMES were formed from the residual oil using a Novozyme SP 435 supported lipase catalyst. An earlier and somewhat similar approach was used by King and Dunford (2002) to enrich phytosterols from deodorizer distillates. One novel aspect of the scheme proposed by Temelli (2009) is the incorporation of the residual protein-carbohydrate fractions for the formation of particles or encapsulates of enriched of the SFE- or SFF-enriched nutraceutical extracts using one of several supercritical fluid based particle production techniques (King 2004a, b).

Several additional bio-refining scenarios based on rice have been conceptualized and reported in the literature. A multi-stage critical-fluid based bio-refining of rice has been described by the Brunner group at TUHH in Germany (Schacht et al. 2008) based on their individual studies of various unit processes constituting the bio-refinery scenario. Milled rice containing ~20 wt% oil of which 8 % are free fatty acids, bioactive ferulate ester–oryzanol, and 80 % lignocellulosic content is initially extracted with $SCCO_2$ and further fractionated to separate the free fatty acid contents from the rice bran oil. The remaining rice straw is then hydrolyzed using carbonated water followed by carbohydrase enzyme treatment to produce monomeric sugars for fermentation to produce a dilute 5–10 wt% ethanol solution. Finally, the aqueous ethanol mixture is fractionated to enrich the ethanol content using $SCCO_2$ to a 99.8 % level.

Similarly, Lee (2012) has envisioned the bio-refining of rice feedstock as shown in Fig. 13.5. Here rice's components are segregated into the bran, hull, germ, and the proteinaceous component (white rice) for further use and transformation. As shown in Fig. 13.5, the brown rice component can be treated via exposure to $SCCO_2$ to enhance its shelf life resulted in a commercially-sold product (likewise for the white rice component). The rice bran can be extracted with $SCCO_2$ to yield a nutritious defatted powder devoid of off-flavor characteristics caused by free fatty acids produced by enzymatic action. The rice bran oil can in principle also be converted to biodiesel by one of the previously described catalytic methods using either $SCCO_2$ or supercritical fluid methanol (SC-MeOH). Other chemical derivatives as pictured in Fig. 13.5 are possible including the production of fatty alcohols previously mentioned in Sect. 13.3 using $SCCO_2$ or supercritical propane.

Yoshida (2012) at Osaka Prefecture University in Japan has developed an ingenious process based on initially on subcritical water hydrolysis to treat and transform both biomass waste, sewage sludge, as well as industrial wastes into an array of useful products. One of these processes involves treatment of sewage sludge that when mixed with water and treated at 300 °C and 10 MPa for (10–20) s. provides a hydrolyzate for further conversion via fermentation. A simplified flow chart of the process is provided in Fig. 13.6 shown below. The hydrolyzate containing a cocktail of sugars, lower carbon number carboxylic acids as well as amino acids can be directed into a fermentation vessel where if it is treated with a methanogen, can produce gaseous mixtures of methane (CH_4) and CO_2. This gas mixtures in the Yoshida scheme are separated by pressure-swing adsorption

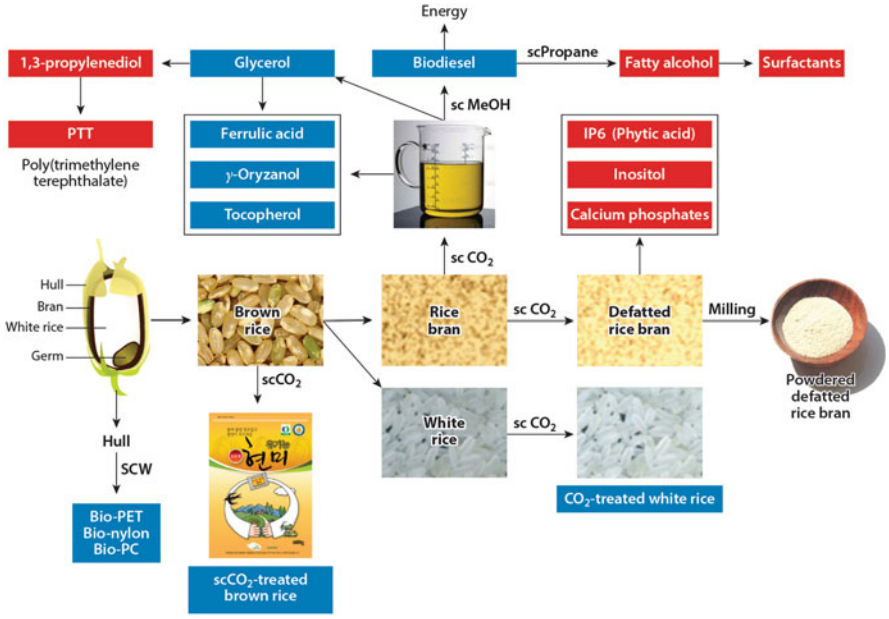

Fig. 13.5 A conceptual rice bio-refinery with integrated supercritical fluid technologies (King 2014)

resulting in purified methane that is then stored in a gas holding tank at 1 MPa pressure. Thus the CH_4 can be used as a source of vehicular fuel for motor bikes and cars. The process is amenable to using other substrates such tofu processing wastes. A description of a total hydrolysis bio-refinery has discussed by King and Srinivas (2009) and a picture of hydrolysis plant run by Rematec Co. in Osaka, Japan is available (King 2012).

Carbonated subcritical water has been used by King et al (2010, 2012) on substrates ranging from grape pomace, corn stover, switch grass to produce aqueous antioxidant extracts and carbohydrate mixtures which can be fermented to bioethanol. A similar approach has also been advocated by Goto et al. (2012) using "hybrid" CO_2-water systems which have been patented. In these cases, the severity and degree of hydrolysis is largely controlled by the temperature of extraction-reaction and the residence time in the processing vessel. The recalcitrance of the biomass to hydrolysis can also be important and can vary considerable for various herbaceous field crops. Carbonated water hydrolysis offers several advantages, namely the avoidance of using mineral acids which require post extraction-reaction neutralization and disposal of the resulting salt by-product. Carbonic acid hydrolytic depolymerization of hemicellulose- and cellulosic-containing biomass can produce substantial amounts of monomeric sugars thereby reducing the amount of expensive carbohydrase enzyme cocktails required to further depolymerize carbohydrate oligomerics to monomeric sugars. SWE can be combined sequentially with higher temperature subcritical water hydrolysis to

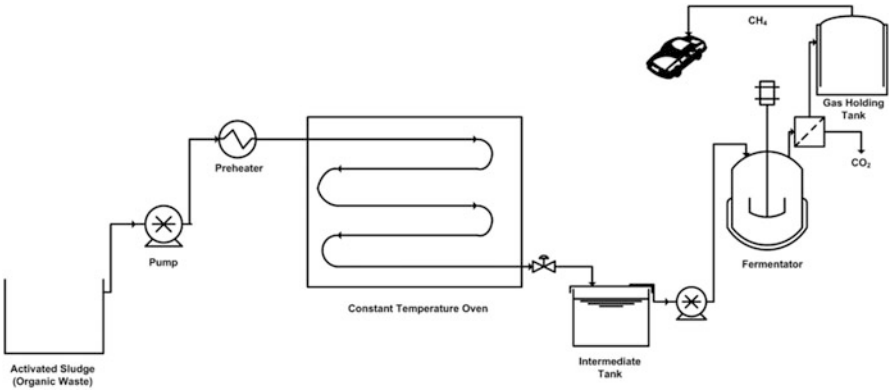

Fig. 13.6 Production of methane from organic waste using subcritical water hydrolysis followed by fermentation of the hydrolyzate mixture

yield both high value nutraceutical mixtures as well as serve as the basis of biorenewable fuel production.

Of considerable current interest is the propagation of algae to produce an oil-barring substrate that has value as a nutritional supplement and a renewable fuel source. Over the past 10 years, the focus has been traditionally on algae oil for use or transformation into a fuel source. Indeed SFE using $SCCO_2$ is one way of isolating the oil from the algae, however assessing the oil from the algae cell can be difficult even when using organic solvents. A typical process flow diagram for the isolation of the high value nutritionally-beneficial pigment, astaxanthin, from *Haematococcus*-derived algae is shown in Fig. 13.7. SFE using $SCCO_2$ is becoming the preferred method of isolating this very valuable human nutritional product ($10,000–12,000/kg) and is best affected at extraction pressures above 80 MPa.

The insertion of this SFE process into overall schemes for using algae-derived oils for integrating SFE into several reaction-dominated pathways for producing biodiesel (FAMES), bioethanol, or biomethane (CH_4) for vehicular fuel as indicated in Fig. 13.8. The proposed reaction pathways Fig. 13.8 makes use the ready availability of high purity CO_2 from fermentation and flue gas coal-fired power generation stages. Such CO_2 sources can feed the algae propagation ponds as well as being used for the SFE of the algae oil. Careful inspection of Fig. 13.8 reveals that this CO_2 can also supplement CO_2-assisted expelling as discussed previously in Sect. 13.3 for isolating the algae oil. The challenge for the critical fluid engineer or scientist is convincing the renewable biofuels community to integrate SFE and SWE into these bio-renewable fuel processes as well as to consider the SFE, SFF, and SFR processes described in this chapter.

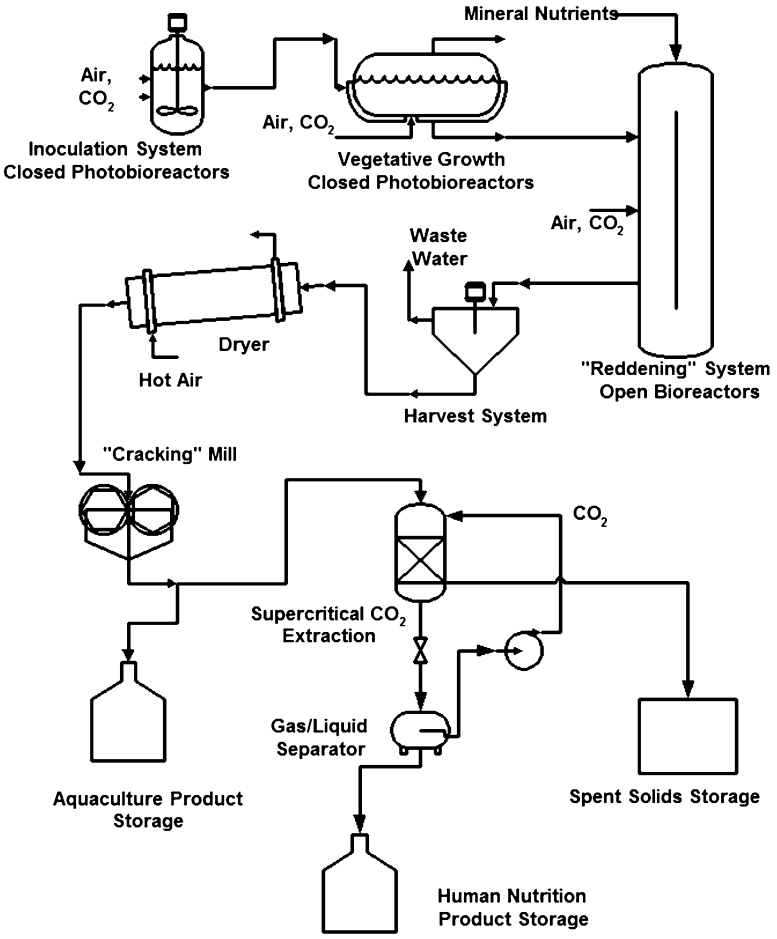

Fig. 13.7 Process flow diagram for the production of natural astaxanthin from *Haematococcus*-derived algae using $SCCO_2$ for pigment extraction

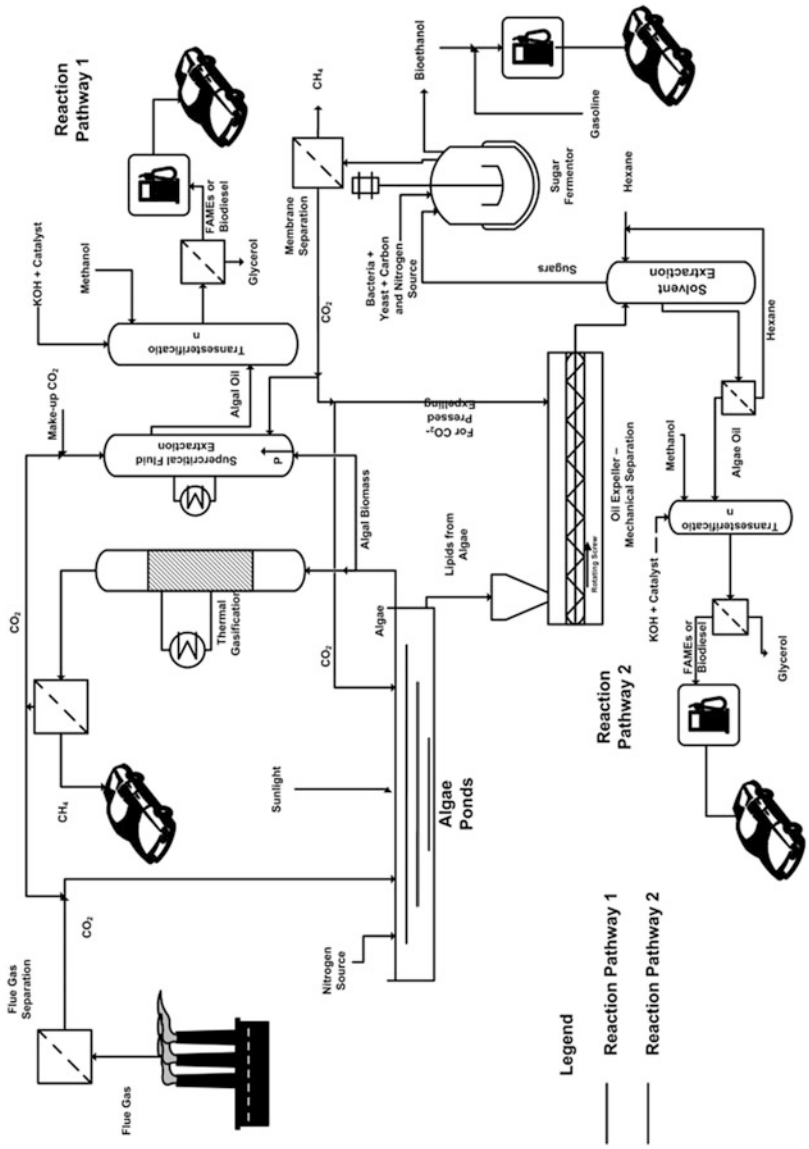

Fig. 13.8 Reaction pathways with integrated SFE for the conversion of algae-derived oil to biofuels incorporating CO_2 recycle

Summary and Conclusions

Additional information on integrated unit operations employing sub- and supercritical fluids can be found in the recent review by King (2014). Integration of SFE as a unit process in bio-refining has been also recently reviewed by Mantell et al. (2013). It is encouraging that researchers the world over are embracing the concept of coupling various critical fluid media and unit processes that was first advocated by King in 2003.

Over the past 25 years, the author has on more than one occasion been queried on the possibility of constructing a SFE plant in close proximity to an alcoholic fermentation facility that produces high purity CO_2 as a by-product. This would seem logical since the opportunity to apply SFE for vegetable or specialty oil extraction and fractionation could be facilitated with this source of CO_2 as well as any of the above mentioned CO_2-based unit processes. The production also of ethanol at such a site facilitates a preferred co-solvent for coupling with CO_2 as documented previously. Today, in the renewable bioenergy field it is envisioned to build coexisting bioethanol and biodiesel production capabilities at the same site. This suggests the possibility of extending the application of critical fluids platform for the production of these two renewable fuels as documented in this chapter. Similarly, it was noted above that $SCCO_2$ could be mixed advantageously with pressurized water for extraction and reaction chemistry. Such a critical fluid-based processing concept supports the use of renewable resources, a sustainability platform, and does so in a "green" environmentally-benign manner.

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Chapter 14
Prospective and Opportunities of High Pressure Processing in the Food, Nutraceutical and Pharmacy Market

Ignacio Gracia

14.1 High Pressure Processing (HPP) in the Food, Nutraceutical and Pharmacy Market

High pressure processing (HPP) is a well established operation nowadays. In the last decades its potential increased from a promising new technology generally focused on natural products extraction with a generalized lack of scientific knowledge and technical skills, to a wide range of specific applications involving catalytic reactions, biomass conversion, particle formation, drug delivery or biomedical applications (Caputo et al. 2013). One of the most promising applications of high pressure processing concerns food, nutraceutical and pharmacy industry. In these fields theoretical, operative and technical aspects are well known nowadays. Thermodynamic and kinetic information is generally available for a large number of compounds and systems; and the behaviour of the process is usually well known and different models can be used to predict and optimize the results.

Simple extracts to final products can be obtained using different isolation strategies such as extraction, concentration, fractionation, impregnation, reaction, precipitation (Perrut 2000; Brunner 2012). The procedure can be performed in single step or multistage extraction, column fractionation or multiphase reactions in devices from cm^3 to m^3. No limitation exists about physical nature of samples - HPP can be used coupled to classical refining or treatments or even a sequence of processes in "one pot" procedure can be performed. As regarding final product production advantages, HPP can obtain very narrow size distribution particles, tuned dosage and delivery capacity and different presentations like microencapsulation that can increase the solubility and reduce the dosage of drugs. In addition, HPP products are well characterized and based in research and innovative processes

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and can quickly meet the new changing customer demands, essential point for marketing requirements. Regarding normative, the usual solvent CO_2 is GRAS solvent, and all scientific basis of HPP lead to their products to follow the new regulations about marketing and labelling like EFSA and FDA requirements (Reutersward 2007).

In spite of advantages regarding production, safety, quality, normative and marketing, the industrial implementation of HHP products is scant, and generally associated to high scale production capacities (Perrut 2000). One of the reasons is that HPP products are generally considered to be simple substitutes of those obtained with classical processes. The latter generally produce products of lower quality and are prone to problems such as use organic solvents, bad or low characterization and lack of scientific research-basis that can differentiate the product and justify its application.

The analysis of this situation from an economical and marketing point of view is that usually the selection of the market niche is wrong, and HPP products should be introduced in a new segment. This fact constrains to clearly identify both strategic and economical reasons for the decision. Unfortunately, in the industrial development of new products are involved marketing and finance strategies, areas usually unknown for researchers. The aim of this chapter is to present some strategies to determine and quantify the industrial potential of HPP processes in the food, nutraceutical and pharmacy market. Furthermore, tools applied to calculate the production costs, the selling price, the basic accounting of the process and some financial ratios required in the final implementation will be described.

14.2 Classical Production Processes and New Market Requirements

The industrial implementation of a new product (Fig. 14.1) is a complex process which is generally a consequence of the interaction between the entrepreneur and the environment to obtain an idea for a new product or application (Gracia 2011). This initial idea must be evaluated in an iterative process to determine whether it meets the market needs in order to discarded or redefined. Once selected, the idea must be developed to determine its viability in an exhaustive procedure that consists of the compilation of a great quantity of information for the elaboration of a *Business Plan*. Once the economical viability is confirmed, other sensitivity tests must be performed in order to consider other external aspects such as global and local situation or even, the interest rate that can affect the profitability of the inversion compared to a bank deposit. Only after these requirements are fulfilled, the process or company can be launched administratively. After financing is obtained, the plant is constructed and the start up can be performed.

Hence, the Business Plan is a key document in this sequence that includes all aspects affecting the setting-up of a company or a new product. It represents a tool

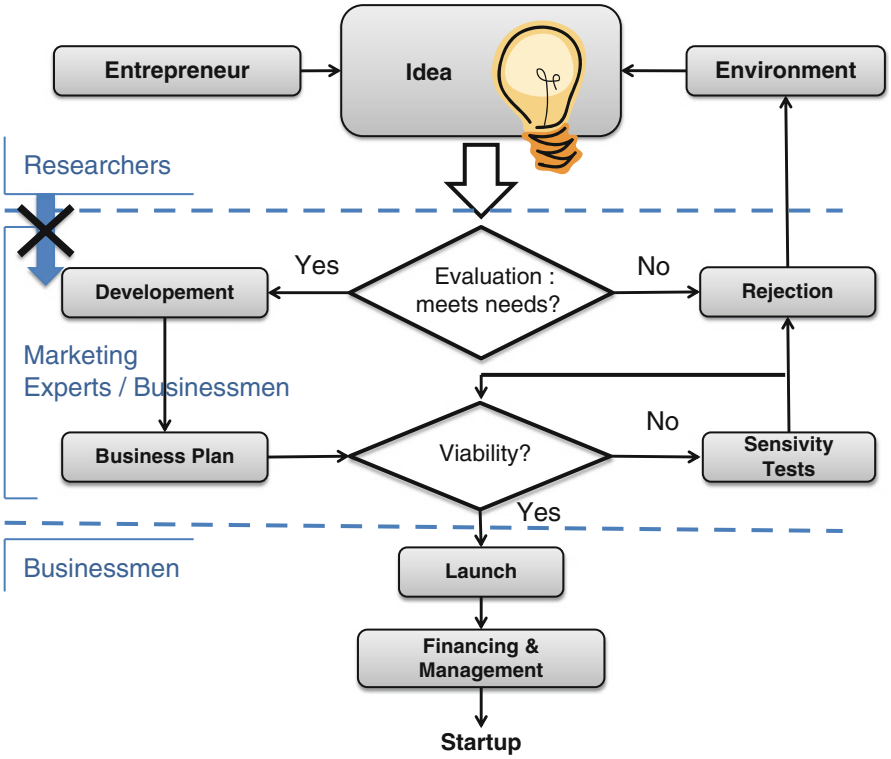

Fig. 14.1 Sequence for the industrial implementation of a new product or company

to analyze any of the different aspects (Fig. 14.2), and to determine the investment; being crucial to check the economical feasibility and to take strategic decisions in any time of the project life. In the Business Plan information is generally organized according to the following items:

1. *Introduction*. Includes background and presentation of the idea, definition of the product and the entrepreneur.
2. *Market analysis*. About environment, sector, customers, competitors and SWOT test.
3. *Marketing Plan*. Targets and market strategies
4. *Organization Plan*. Includes facilities, production process, suppliers and human resources.
5. *Legal and financial aspects*. Administration and start up Schedule.
6. *Financial Plan*. Includes investment, depreciation, costs analysis, income, financial ratio indexes and breakeven point.

Researchers and people involved in the scientific and technical development of a new process should participate in items #1, #4 and #6. The special characteristics of HPP and even those concerning food, nutraceutical and pharmacy market, make

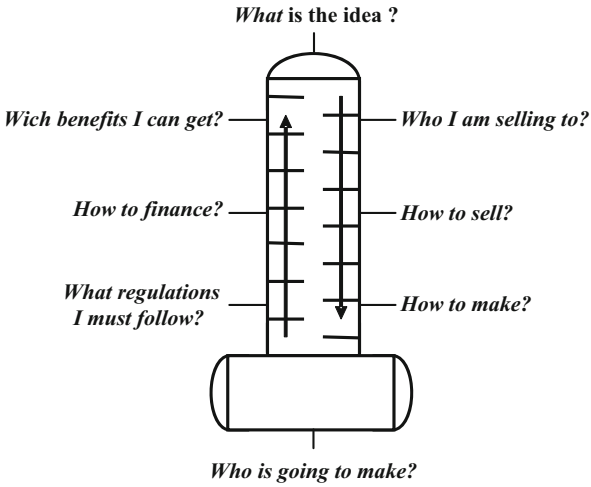

Fig. 14.2 Questions to be answered in a Business Plan

essential the contribution of scientists in practically the whole process. However, as indicated in Fig. 14.1, their participation is limited to the inner steps; marketing experts and businessmen being those who usually take strategic decisions about the implementation of new products or technologies.

This situation is one of the most important reasons for the scarce implementation of HPP. Thus, scientists should increase their knowledge about the process, especially in marketing and financial skills, in order to be able to make understanding and defending their alternatives in fields where the introduction of new products is difficult (Gracia 2011).

14.3 Classical Processes and New Market Requirements

Conventionally, the extraction and production of bioactive compounds has been attempted by hydrodistillation, low pressure solvent extraction, maceration (Herrero et al. 2006; Gil-Chavez et al. 2013; Fernández-Ronco et al. 2013a), or any other conventional technique. Economic analyses based on classical processes are quite simple because they are frequently well defined, are based on high-scale continuous operation, and are focused on products which are widely accepted by the market and present low fluctuations in their price. In this case the factors affecting profitability are basically *fixed* and *operating costs or manufacturing costs*. However, new products, especially those concerning health, should deal with new market requirements: high quality, high knowledge about chemical composition, high adaptation to changes in market demand, innovative processes and products, low processing time, small scale, environmental requirements and/or exclusivity (Gunasekaran and Ngai 2012; Mark-Herbert 2004; Perrut 2000).

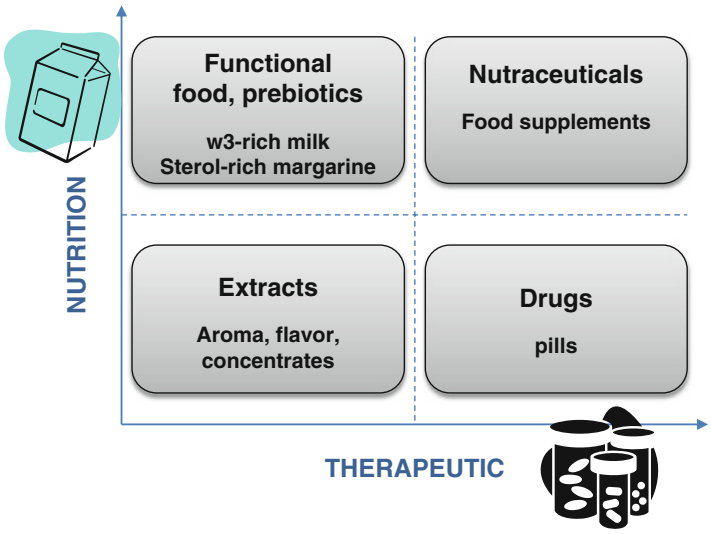

Fig. 14.3 New products in the food, nutraceutical and pharmacy/cosmetic market

In spite of HPP or Supercritical Fluid Technologies viability for the new market needs, the industrial implementation of supercritical-based products (Fig. 14.3) is difficult (Brunner 2012; Perrut 2000; King et al. 2011). One of the reasons is the difficulty to determine the investment costs based on different production scales. What it is more, it is especially difficult to determine the product's price depending on its composition in the possible formulations from food to pharmacy presentations, which are generally new for the market (Gunasekaran and Ngai 2012).

When studying the economical feasibility of this technology, HPP is erroneously considered to be like conventional processes; analyses are generally performed based on the statement of costs, which consider HPP products like simple substitutes of a conventional one, with a similar price. Low prices, in addition to high pressure operation facilities are usually responsible for the fact that HPP is wrongly disregarded as a viable alternative. In these cases previous literature research reported that the cost of manufacturing was higher than or similar to those corresponding to the selling price of the compared product (Prado et al. 2010; Pereira and Meireles 2010; Mezzomo et al. 2011). This view logically constrains the industrial application of HPP to high-scale processes like coffee decaffeination, sesame oil or cork production (Lee 2012; Brunner 2012; King et al. 2011) indicating that other factors must be taken into account to better appreciate and use the product with higher selling prices (Perez-Silvestre 2010). As example, The UMax plant, constructed in 2005 by Natex, produces about 2,000 t per year of sesame oil by using two 3,000 L batch extractors (King et al. 2011).

New attractive opportunities for natural products processing and extraction/fractionation processes have been appearing lately. In the HPP preparation of high value products like food supplements and nutraceuticals, the "natural"

character of the preparation mode has a high marketing value, the cost being much less sensitive than for food or perfumery products (Perrut 2000). The above indicates that HPP is able to obtain specialties and fine chemical-like products, compared to classical commodities-like products, and this increase of quality must be conveniently justified by researchers using a business plan analysis to change their market niche. In the food, nutraceutical and pharmacy rapidly expanding market there are opportunities for health, well being, boutique, smaller-scale producers to focus on the top end of the market (Hughes 2009; Mark-Herbert 2004).

14.4 Food Regulations and Challenges of HPP

In a growing number of countries, most organic solvents have been banned for food products extractions. These kinds of regulations will ease HPP to develop in relation to issues regarding consumer and environmental protection (Perrut 2000). The EU (European Food Safety Authority, EFSA) and the US (Food and Drug Administration, FDA) have been involved in international actions toward sustainability, and have enacted national policies to implement the commitments. The EU has endorsed more mandatory measures than the US in terms of environmental practices. In addition to government regulations, sustainability in the food sector is promoted through private standards amongst processors, producers and retailers. These private standards have international trade implications and present several legal challenges that range from threats to the viability of the food regulatory system to practical concerns relating to capacity, contract compliance and the risks of careless standard-setting (Roberts and Leibovitch 2011).

The challenge of Health claims. Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods clearly state that "*Nutrition and Health Claims on Food must be substantiated by scientific evidence*". A *health claim* is defined as "any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health". The specific health claim, dealing with *reduction of disease risk claim*, is defined as "any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease".

In practice, three main types of health claims are included in the Regulation, as referred to in Articles 13 and 14. Furthermore, there is one group of claims, referred to as Article 13.5. (Claims based on newly developed scientific evidence and/or which include protection of proprietary data). The health claims are:

- Reduction of disease risk claim (Article 14 claim)
- Health claims referring to children's development and health (Article 14 claim)
- Other health claims (Article 13 claim).

Functional foods have been a topic since the mid-1980s. Over the years a significant amount of scientific evidence showing physiological effects of other

substances, i.e. non-nutrients, has been published. Two groups of "other substances" are plant sterols/stanols and their esters and probiotics. The regulation is now opening up for the food business (Reutersward 2007). HPP products are obtained generally via scientific research and follow the new normative regarding *marketing*, *composition and labeling*, also indicated in the Codex Alimentarius (1999). This fact is an additional advantage for the implementation of these products in the high quality market.

14.5 Opportunities for HPP

Economic evaluations based on the Business Plan strategy are based on a methodology in which the process is firstly evaluated by a *SWOT analysis*. This method takes the information from an environmental analysis and separates it into internal (*strengths* and *weaknesses*) and external issues (*opportunities* and *threats*), to obtain the SWOT matrix, as shown in Fig. 14.4.

The elements of the SWOT matrix can be defined as:

- *Strengths*: characteristics of the business or project that give it an advantage over others;
- *Weaknesses*: characteristics that place the team at a disadvantage relative to others;
- *Opportunities*: elements that the project could exploit to its advantage;
- *Threats*: elements in the environment that could cause trouble for the business or project.

The internal *strengths and weaknesses* are things the firm has full control of. On the contrary, the external *opportunities and threats* deal with influences and conditions out of the organization full control. Table 14.1 gives information about different factors to be usually considered.

For HPP in the food, nutraceutical and pharmacy market, some specific topics can be considered (Gil-Chavez et al. 2013; Mhurchu and Ogra 2007; Steenhuis et al. 2011; Hughes 2009):

Strengths:

- patents;
- costs advantages from proprietary know-how;
- exclusive access to high grade natural resources;
- collaboration with technological research centers.

Weakness:

- high costs structure;
- lack of access to distribution channels;
- limited economic resources.
- new and growing market;

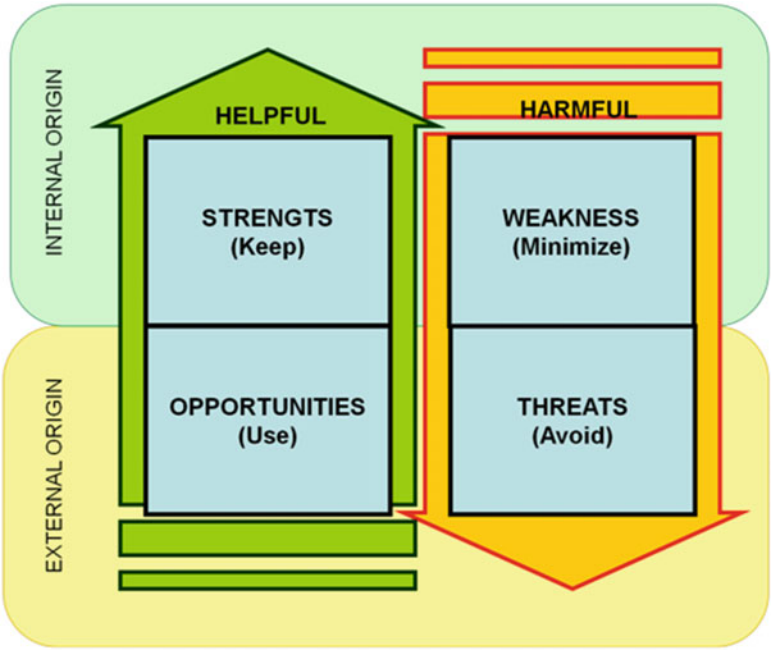

Fig. 14.4 Elements of the SWOT matrix

Opportunities:

- unfulfilled customer need;
- new technologies;
- regional economic and social development;
- new food regulations and *health claims* normative.

Threats:

- strong competitors;
- emergence of substitute products;
- shift in consumer tastes;
- new regulations;
- high Taxes.

14.6 Business Plan Advantages for HPP

Researchers have reported several advantages for the application of HPP in the food, nutraceutical and pharmacy market. As previously cited, all of them indicate the high quality of HPP products compared to classical ones, and consequently the convenience of improving their market niche (Pereira and Meireles 2010;

Table 14.1 Environmental factors to be considered to perform the SWOT test

Internal	
Finance	Is the organization profitable?
Business processes	Are these efficient?
Communication	Are there effective lines of communication?
Management & Leadership	What are the management and leadership styles?
Cost	What unique resources do you have access to?
Competitive Advantage	What do you do better than anyone else?
Resource	What resources do you have access to?
Sales	What are the factors to increase or decrease sales?
External	
Competitors	What does their SWOT look like?
	Are competitors doing or changing anything?
Markets	Are there any new markets for our products?
	Are there any new markets for our services?
	Is there any a new product or services for our market?
Political	Is there any government legislation?
Demographics	Are there any age or socio-economic factors?
Technology	Are there any new technological breakthroughs?
	Is changing technology giving competitors the edge?
Barriers	What barriers exist in the marketplace?
Trends	Are there any trends or patterns in the market?
	Are industry, technology or cultural trends changing?

Fernández-Ronco and Gracia 2011; Fernández-Ronco et al. 2013b; Montanes et al. 2012; Bravi et al. 2002).

Table 14.2 shows examples of SWOT matrix details for HPP processes in the field considered in this chapter. As it can be observed, the main opportunities concern the high quality of products and their increased added value leading to their placement in different segment or market niche with higher selling prices. From a marketing point of view the exclusivity and the "natural" character of HPP is a point to be considered for, representing a clear "top product" strategy.

The rising consumption of gourmet or healthy food all around the world is due to people awareness on health improvement through diet. Nowadays, the demand of this type of food is increasing rapidly, justifying the production of new added-value products and the increase of their selling price (Mhurchu and Ogra 2007; Steenhuis et al. 2011). On the other side, HPP plants are versatile and could be used for different products and processes (for instance supercritical fluid chromatography) with low investments, especially for low-scale production processes (Perrut 2000).

The "high scientific level" of HPP produces strengths related to high characterization and standardization of products, leading to interesting options to follow healthy or functional food normative about marketing and labeling for the corresponding food claims. On the other hand, all solvents used in the process (i.e. CO_2, ethanol, water) are recognized as GRAS; In particular carbon dioxide has

Table 14.2 SWOT analysis for HPP processes (Montanes et al. 2012; Fernández-Ronco et al. 2013b)

Opportunities	*Threats*
Interest of companies in marketing	Low control in final distribution
Increase the added-value of products	Quite similar to conventional oleoresin
Open the market of high value products or "gourmet" quality	Low price control
Increase concern for "healthy food"	
Versatile operation plants	
Strengths	*Weaknesses*
Product standardization	Irritation at high concentrations
Stagger company in stages	Preparation of different mixtures
Available physical resources	Not constituted company
Available financial resources	Lack of skilled workers for management of HPP plants
Low environmental impact	
GRAS solvents	
People awareness on health improvement though diet	
Low cost raw material	

a low environmental impact, can be recirculated and usually compressed carbon dioxide comes from ammonia production, therefore, no transfer to atmosphere and thus no ozone depletion are involved (Montanes et al. 2012). In addition, the cost of the raw material is usually low, thus contribution to the global economics of the process is very low or even negligible (Perrut 2000; Montanes et al. 2012).

The main threats and weaknesses are related to the way industries comprehend supercritical technology considering their products just simple substitutes. The low control in distribution channels can influence the incorporation of the products into the market, making difficult to control the product price (for instance, lactose prices increased a 300 % between 2004 and 2007 as shown by Montanes et al. 2012). This threat, coupled with the concern about customers capability to differentiate the new product from the conventional ones will affect the marketing strategy, which should be focused on highlighting the benefits and differences of the new products (Rosa and Meireles 2005).

14.7 Economic Analysis for HPP: From Laboratory to Industrial Scale

The SWOT test is not a simple description of items. It must identify the required actions to *use the opportunities*, *keep the strengths*, to *minimize or correct the weakness* and to *avoid of face threats* (Fig. 14.4).

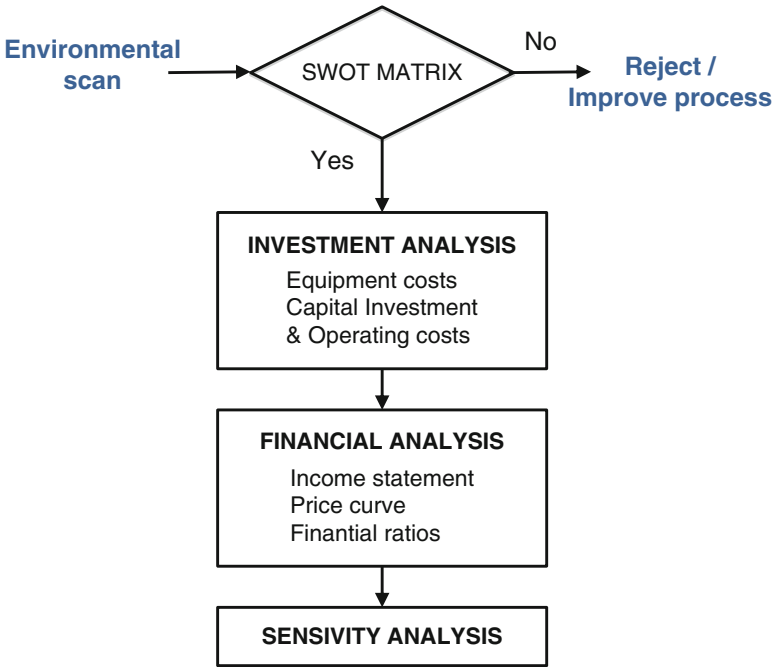

Fig. 14.5 Flow diagram of the economic analysis based on the business plan strategy

As a whole, the evaluation of the SWOT matrix allows determining the interest of the process or the necessity to enhance different aspects prior to its detailed economic evaluation (Fig. 14.5). If the conclusion from the SWOT matrix is positive, the following step comprises the *investment analysis* which includes the calculation of the equipment cost required for the process and then the total capital investment of the plant. The elaboration of investment analysis represents the basis for the process economics due to costs are calculated in this step. Figure 14.6 gives information about the different steps and requirements to complete the investment analysis.

14.7.1 Investment Analysis

The first part of the investment analysis requires *experimental or laboratory information* about the kinetics and phase equilibrium of the process. Mass transfer parameters are generally obtained by fitting experimental data to different models proposed in the literature (Albuquerque and Meireles 2012; Pereira and Meireles 2010).

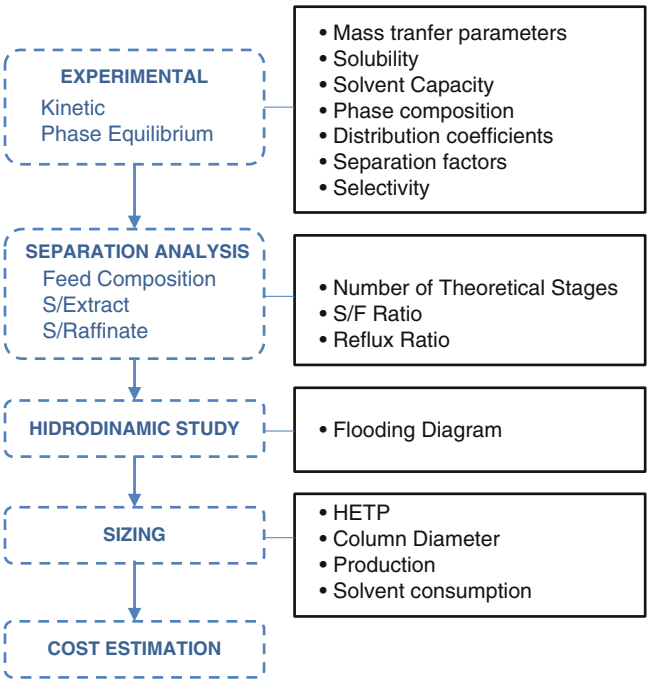

Fig. 14.6 Investment analysis. Sequence for determining the equipment cost

Mass Transfer coefficients can be expressed in terms of dimensionless correlations that consider the influence of natural and forced convections, or only forced convection simultaneously (Mezzomo et al. 2013):

$$Sh = 2 + 1.1\, Re^{0.6} Sc^{01/3} \tag{14.1}$$

adequate for $3 < \mathrm{Re} < 3{,}000$ and $0.5 < \mathrm{Sc} < 10{,}000$.

$$Sh = 0.38\, Re^{0.6} Sc^{01/3} \tag{14.2}$$

valid for $2 < \mathrm{Re} < 70$ and $2 < \mathrm{Sc} < 11$.

The knowledge of phase equilibrium data is of fundamental importance for the design of simple or multistage gas extraction, as it provides the thermodynamic basis for the separation process analysis. Phase equilibrium data yield information concerning the mutual solubilities (especially those in the gaseous phase), solvent capacity, and compositions of the coexisting phases, distribution coefficients, and selectivity. The solubility of multi-component mixtures in the gaseous phase gives information about the amount of solvent, and the mechanical energy requirements to recycle this amount of solvent, necessary to achieve a desired separation task (Brunner and Machado 2012; Pereira and Meireles 2010).

Equilibrium data can be correlated from simple generic correlations (Valle and Aguilera 1988) to Equations of State (EoS) calculations (Fernández-Ronco et al. 2011b). For the fractionation of multi-component mixtures, it is convenient to express these data in terms of *separation factors* or *distribution coefficients*, where complex systems like fatty acids or oleoresins can be reduced to quasi-binary systems consisting of two pseudo or key compounds and calculate the selectivity (Brunner and Machado 2012; Fernández-Ronco et al. 2011a). The distribution coefficient K_i can be calculated as the ratio of the concentration of component i in the gas phase, y_i, to its concentration in the liquid phase, x_i.

$$K_i = \frac{y_i}{x_i} \tag{14.3}$$

The *separation analysis* needs information concerning the distribution coefficients K_i of all the components of the multicomponent mixture. The distribution coefficients K_i, described on a solvent-free basis, provide information about the phases where the components of a multi-component mixture are preferably enriched. The distribution coefficients K_i indicate what components are preferably enriched in the extract ($K_i > 1$) or in the raffinate ($K_i < 1$). The feasibility to separate key compounds HVC and LVC (*high volatile pseudocompound* and *low volatile pseudocompound*, respectively) can be determined by calculating the separation factors between these two pseudocompounds as:

$$\alpha_{HVC/LVC} = \frac{K_{HVC}}{K_{LVC}} = \frac{y_{HVC} \cdot x_{LVC}}{x_{HVC} \cdot y_{LVC}} \tag{14.4}$$

The higher the separation factor is, the easier the separation of the components is. With HVC/LVC equal to 1, no separation of the components HVC and LVC is possible. Figure 14.7 shows de distribution coefficients of oleorresin capsicum at different temperatures (Fernández-Ronco et al. 2011a).

The last step of the separation analysis consists of determination of the most suitable operational conditions. After mass transfer and thermodynamic information is available, several processes can be performed in order to determine the correct sizing and scale-up of the industrial plant according to the scheme presented in Fig. 14.6. For determining the most suitable operational conditions to carry out the separation analysis, it is important to find a compromise between the separation factor and the solubility in the supercritical phase, because these variables are related to the number of theoretical stages and the amount of CO_2 needed to achieve the separation. Within the feasible area, different conditions could be selected. These conditions correspond to the interception point of solubility and separation factor lines at each constant temperature. Comparing the results for all of them, it is easy to establish the most suitable operational conditions as those where solubility and separation factors are bigger (Fernández-Ronco et al. 2013b).

As indicated before, the importance of the separation analysis is due to its ability to determine the optimum operating conditions for the separation process. In the

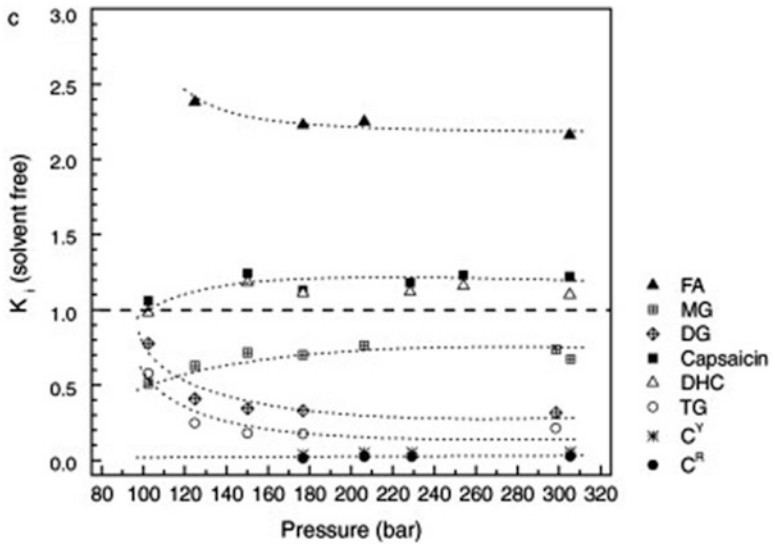

Fig. 14.7 Distribution coefficients of oleorresin capsicum at different temperatures (Fernández-Ronco et al. 2011a)

case of a separation column, the optimization can be performed by correlating the number of theoretical separation stages (NTH), the solvent-to-feed (S/F) ratio and the reflux ratio. For this purpose different short-cut methods can be used, and the influence of some variables like the effect of extracts and feed composition should be studied.

The selection of a short-cut method can have a big influence on the number of stages obtained. While McCabe–Thiele method only considers the separation as a binary or pseudo-binary one, neglecting the effect of the solvent, Ponchon–Savarit method, using the Jänecke diagram, considers the solvent effect and analyses the separation problem with a pseudo-ternary approach (Fernández-Ronco et al. 2011a; Brunner and Machado 2012).

To conclude the analysis of the effect of feed composition on the separation problem, the solvent-to-extract ratio and the solvent-to-raffinate ratio can be studied as indicators for operation costs. Since both products can be commercialized, they incorporate all process costs (Budich and Brunner 2003). Figure 14.8 shows the influence of the feed composition on the solvent-to-extract and on the solvent-to-raffinate ratios, respectively, in the supercritical fractionation of capsicum oleoresin (Fernández-Ronco et al. 2011a).

Number of stages and solvent-to-feed ration as a function of reflux ratio. The method proposed by Billet (1995) to minimize the column volume uses the reflux ratio as the independent variable while the modification of Billet's method to countercurrent gas extraction uses the solvent-to-feed ratio as the independent variable. Besides the number of stages, the solvent-to-feed ratio is the most important design variable to optimize the size of countercurrent columns, because

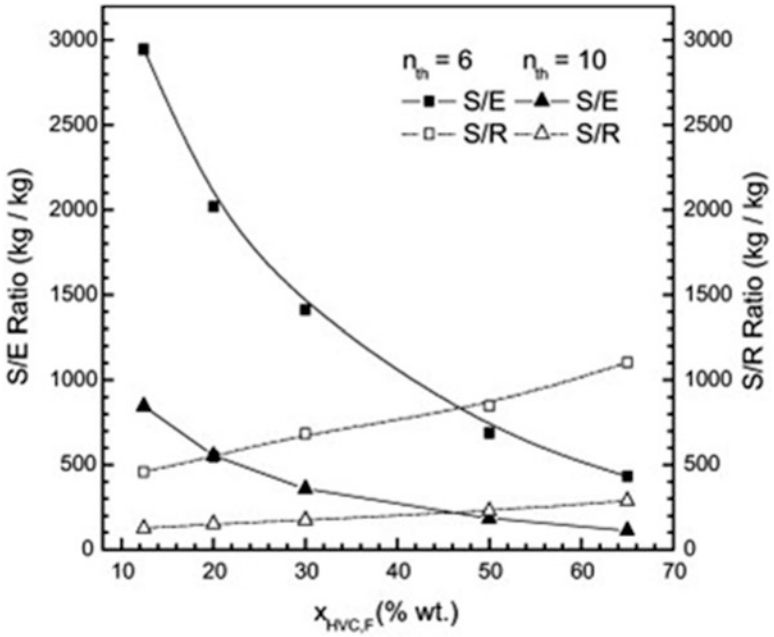

Fig. 14.8 Influence of the feed composition on the solvent-to-extract and on the solvent-to-raffinate ratios in the supercritical fractionation of capsicum oleorresin (Fernández-Ronco et al. 2011a)

this ratio is a direct measure of the solvent consumption and hence of the column diameter (capital), the compression costs and the operation costs (Brunner and Machado 2012). Figure 14.9 gives the corresponding number of stages and solvent-to-feed ration as a function of reflux ratio for the same conditions as those shown in Figs. 14.7 and 14.8.

Calculation of HETP. The mass transfer performance in countercurrent columns is commonly presented in terms of HETP. Especially of interest in multistage gas extraction are the relations between the HETP and the process design variables such as solvent-to-feed ratio, solvent-to-liquid ratio, solvent and liquid mass velocity, reflux ratio, and composition. The influence of design variables on HETP values can be used to estimate the effect of scale-up on the mass transfer efficiency of extraction columns. The knowledge of both HETP and the number of theoretical stages required to accomplish the separation, allows the determination of the height of the column.

Determination of column diameter. A crucial design parameter of a separation process is the minimum diameter of the column at which no flooding can occur in current conditions. Flooding occurs when the maximum hydrodynamic capacity of a countercurrent column is exceeded and depends on pressure, temperature, and geometry of the column. Among the hydrodynamic variables, kind of packing characteristics, and physical properties influencing flooding, the density

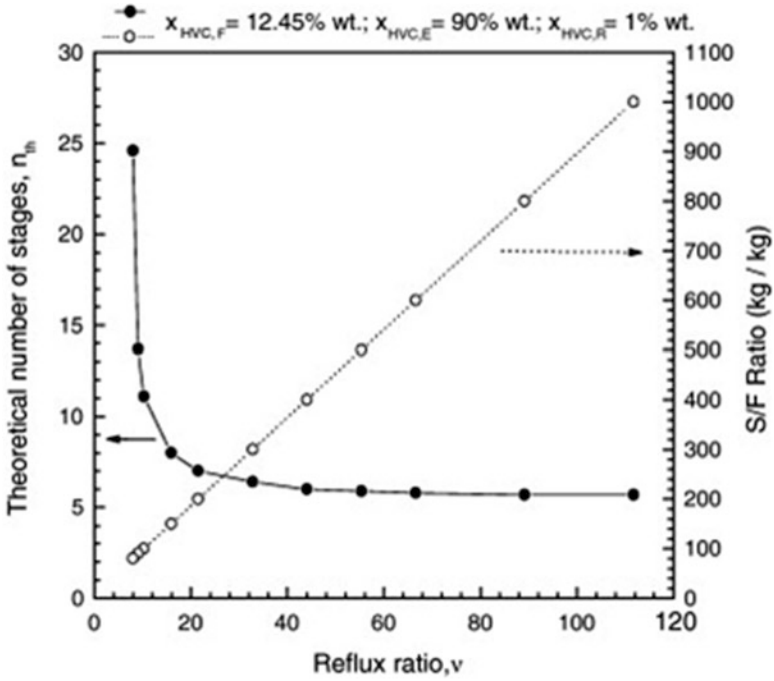

Fig. 14.9 Calculation of the theoretical number of stages using Janeke method (Fernández-Ronco et al. 2011a)

difference of the coexisting phases plays a central role as a limiting factor to assure countercurrent flow (Brunner and Machado 2012).

A generalized and non-dimensional figure, which reports the flow parameter (Φ) and the modified Froude number of the gas phase (Fr_G) as described by Stockfleth and Brunner (2001), is used for calculations (see Fig. 14.10). The flow parameter is described in the literature as dependent only on the solvent-to-feed ratio (Brunner and Machado 2012; Fernández-Ronco et al. 2013b; Pereira and Meireles 2010).

Once the operative conditions and size of the main equipment are determined, it is possible to perform investment analysis by calculating the equipment cost, in addition to the capital investment and the operating costs. Due to their importance, this topic will be discussed in a separate section of the chapter.

14.7.2 Financial Analysis

Once the investment analysis is completed, the remaining steps of the scheme in Fig. 14.5 can be performed. The evaluation of the i*ncome statement*, the determination of the *financial ratios*, and the establishment of the *price curve* included in

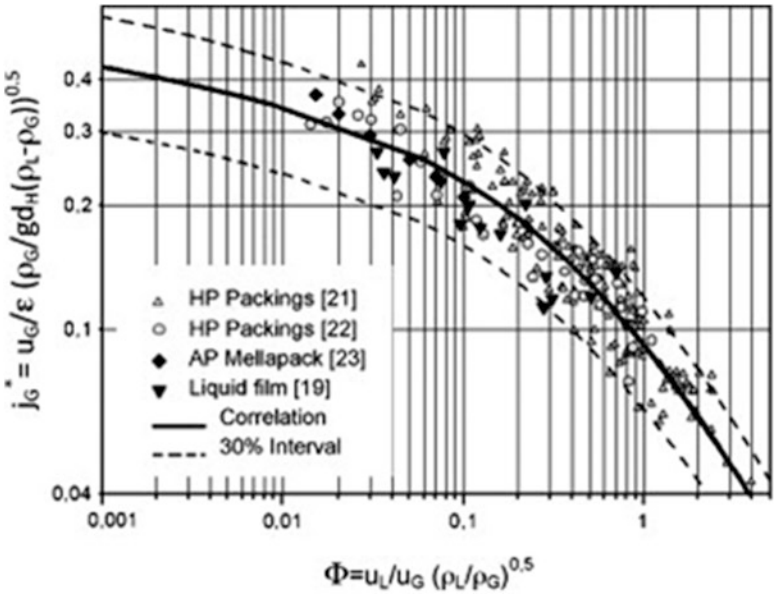

Fig. 14.10 Flooding diagram. AP: high pressure; P: atmospheric pressure. Adapted from Stockfleth and Brunner (2001), Fernández-Ronco et al. (2013b)

the financial analysis offer all the information required to accept or discard the project based on economic reasons.

Income statement. The income statement displays the revenues recognized for a specific period, and the cost and expenses charged against these revenues, including e.g. depreciation and amortization of various assets, and taxes (Helfert 2001). Therefore, the transactions presented in the income statement are of particular interest to show investors a snapshot of all current assets and liabilities of the company.

Financial ratios. In any financial analysis, the determination of financial ratios should be carried out to extend the results from the income statement in terms of *quantitative parameters* that can accept or reject the proposals (Mendes et al. 2002; Fernández-Ronco et al. 2013b; Montanes et al. 2012). Table 14.3 compiles the most representative financial ratios of economic analyses, including the equation, definition and healthy value for each index. Values of those ratios above their corresponding healthy values indicate a good financial situation of the company, thus increasing the interest of possible investors.

In particular, the profitability ratios, namely ROI (return on investment), ROS (return on sale) and ROE (return on equity), measure the use of the assets and the control of the expenses to generate an acceptable rate of return for the company. High values of ROE show the capacity of this business to generate cash and hence, the advantages of investing capital in the project.

Table 14.3 Financial ratios of economic analyses

Financial ratio	Equation	Definition	Healthy value
Return on Equity (ROE)	$\text{ROE} = \frac{\text{Net Income}}{\text{Shareholder's Equity}}$	Profit generated with the money that shareholders have invested	>13%[a]
Return on Investment (ROI)	$\text{ROS} = \frac{\text{Net Income}}{\text{Sales}}$	To compare the efficiency of a number of different investments	>15 %
Return on Sales (ROS)	$\text{ROS} = \frac{\text{Net Income}}{\text{Sales}}$	Profit produced per euro of sales	>10 %
EBITDA to Sales	$\text{EBITDA to Sales} = \frac{\text{EBITDA}}{\text{Revenue}}$	Company's operational profitability by comparing its revenue with earnings	>10 %
Solvency	$\text{Solvency} = \frac{\text{After TaxNet Profit+Depreciation}}{\text{Long Term+Short Term Liabilities}}$	Company's ability to meet long-term obligations	>0.2
Acid Test (Quick test)	$\text{Acid Test} = \frac{\text{(Cash+Accounts Receivable+Short Term Investments)}}{\text{Current Liabilities}}$	Enough short-term assets to cover its immediate liabilities	>1
Cash Ratio	$\text{Cash Ratio} = \frac{\text{Cash Equivalents+Marketable Securities}}{\text{Current Liabilities}}$	Determines how quickly the company can repay its short-term debt	
Debts to Capital	$\text{Debts to Capital} = \frac{\text{Total Debts}}{\text{Shareholder's Equity+Debt}}$	Ability to absorb asset reductions without jeopardizing the interest of creditors	Low
Credit worthiness	$\text{Credit worthiness} = \frac{\text{Available Assets}}{\text{General Liabilities}}$	Company's ability to meet debt obligations	High
Interest coverage	$\text{Interest coverage} = \frac{\text{EBIT}}{\text{Interest Expense}}$	How easily a company can pay interest on outstanding debt	>1.5
Manoeuvre fund (Working capital)	$\text{Manoeuvre fund} = \text{Current assets} - \text{Current Liabilities}$	Company's efficiency and its short-term financial health	High
Break-even-Point		The point at which a business begins to make profits	
Margin of safety	$\text{Margin of safety} = \frac{\text{Sales}}{\text{Break even Point}}$		>1.1

Source: http://www.investopedia.com/terms/

[a]Based on comparative interest for bank deposit

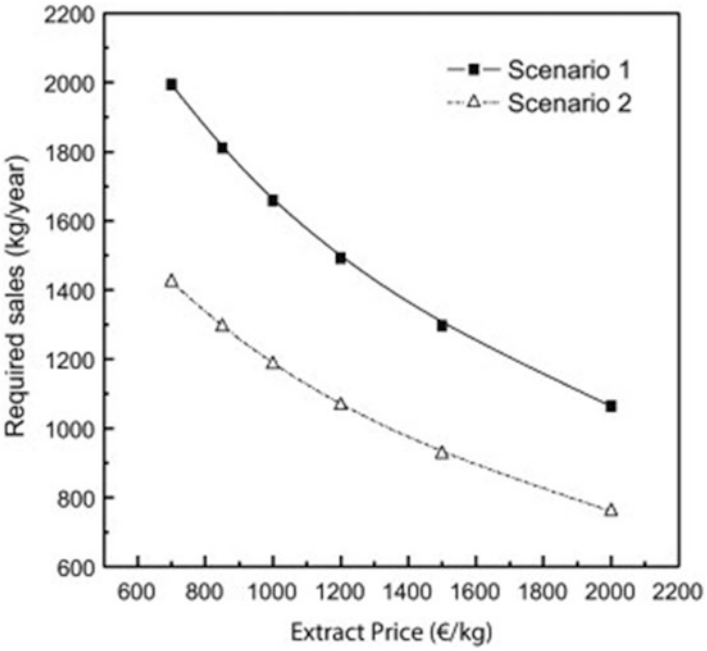

Fig. 14.11 Price curve for the supercritical fractionation of OR_{Cap} assuming a raffinate price of 150 €/kg. Production: 10 t/year (Fernández-Ronco et al. 2013b)

Price curve Determination of price is crucial for a project. In this step all previous business plan strategies to improve the market niche of HPP processes must be taken into account. In fact, the price is not a single point, but a relationship depending on the sales required to meet total cost, namely the *breakeven point*. This relationship is the price curve which gives highly valuable information to define the *sales target* for any given fixed price, or about the admissible *price reduction* in the case of fluctuations in the market demand. As can be seen from Fig. 14.11, a higher product price produces a decrease in the required sales.

14.7.3 Sensitivity Analysis

To determine the influence that possible divergences or changes in the basis of the financial plan have on the economics of the process, a sensitivity analysis can be developed. Sensitivity analysis is also desired to assure the decision about the project. In fact, many of the methods proposed to calculate the total capital investment, e.g., from equipment cost, can produce underestimations in the final investment which can lead to wrong decisions. In this test, some items of the financial analysis are under or overestimated, and the effect of this change on some financial indexes is observed, corroborating the strength of the business

Table 14.4 Evolution of the Pay-back value as a function of the underestimation of different items (Fernández-Ronco et al. 2012)

	Pay-back (years)	
	First scenario	Second scenario
Original situation	4.03	3.49
Fixed capital investment (+10 %)	4.32	3.75
Operating costs (+10 %)	4.09	3.54
Interest rate (+5 %)	4.50	3.85

plan strategy (Mendes et al. 2002; Fernández-Ronco et al. 2012; Montanes et al. 2012). Fixed capital investment, operating costs and interest rate are the three items usually considered for these calculations. In the supercritical fractionation of liquid oleoresin capsicum (Fernández-Ronco et al. 2012), the interest rate was determined to be the most affecting parameter on the pay-back and ROE indexes, as indicated in Table 14.4 for the Pay-Back values. Melo et al. (2014) determined that an accurate definition of the separation vessel pressure has a significant impact on COM (manufacturing cost, USD/Kg extract) value, and that a 25 bar difference in this parameter can lead to a COM increase of 25 %. The effect of CO_2 recycling on the compression costs can increase the total cost of the production with 59 % (Mendes et al. 2002).

14.8 Costs Estimation

This section gives an overview of the main correlations used to perform cost estimation in HPP processes.

14.8.1 Equipment Cost

Methods to estimate high pressure equipment cost are usually based on correlations which link a specific characteristic of the equipment, e.g. heat exchangers area, with its final price. These correlations generally produce underestimation of the cost due to the lack of inflation updating, so that cost must be always updated. The accuracy of these methods depend on the specific type of equipment, and their estimations can drastically change according to the working pressure, type of operation, degree of automation, mode of CO_2 recycling, etc. Therefore, the rather precise method for determining process equipment costs is *directly from suppliers* (Fernández-Ronco et al. 2013b; Alvarez et al. 2009).

14.8.2 Capital Investment

The capital investment has a special importance in the evaluation and development of projects. In fact, it represents both the amount of money which must be supplied for the manufacturing and plant facilities, namely *fixed capital investment* (which can be subjected to amortization), and that required for the operation of the plant, known as *working capital*.

The fixed capital cost is the sum of *direct costs* plus *indirect costs*. The *direct costs* include the main equipment, instrumentation, piping, the auxiliary installations for the production of utilities, such as steam, and the construction of building and the internal structures. The *indirect costs* embrace the engineering expenses and the startup of the plant.

The percentage of delivered-equipment cost is a method to calculate the total capital investment. In this approximation the items included in the total direct plant cost, as well as those from the total indirect cost, are then estimated as percentages of the delivered-equipment cost. Additional components of the total capital investment such as contractor's fee, contingency and working capital are based on average percentages of the total direct and indirect plant cost or on the total capital investment.

The percentages used should be determined on the basis of the type of process involved and its complexity. For HPP those percentages reported by Peters and Timmerhaus (1991) have been used for a fluid processing plant. All expenses directly connected with the manufacturing operation, which can be directly imputable to the fabrication of the product, are included in the *operating costs*. These expenses include raw materials, operating labor, utilities, plant maintenance and repairs as well as taxes (Fernández-Ronco et al. 2013b).

Fiori (2010) estimated the *investment cost* of a supercritical plant by using the formula proposed by Lack, which is valid for extractors in series (Lack et al. 2001):

$$\text{Investment cost} = 1.0163 \ln(\text{VT}) - 4.9147 \tag{14.5}$$

where *VT* is the total extraction volume (L). The formula was derived in 2001 and the actual investment cost must be updated assuming an average increase of the prices, i.e. 3 % per year (Fiori 2010).

It has been shown that the cost of a plant increases with capacity according to the following equations (Shariaty-Niassar et al. 2009; Perrut 2000; Peters and Timmerhaus 1991):

$$\text{FCI}_2 = \text{FCI}_1(\text{RM2}/\text{RM1})^{0.6} \tag{14.6}$$

where *FCI* represents the fixed capital investment and *RM* the raw material (kg/year).

$$FC_2 = FC_1 \left(\frac{V_{EE2} fCO2_2}{V_{EE2} fCO2_1} \right)^{0.24} \tag{14.7}$$

where *VE* is the extractor volume (m^3) and f_{CO2}−the CO_2 flow rate (kg/h). This formula applies for a wide range of sizes and applications.

Direct costs The direct costs (DC) in a SFE unit cover (30–70) % of total production expenses (Rosa and Meireles 2005). This cost rises with the price of raw material (CRM), labor (CL) and the utility (CL), while decreases with the extraction time. Thus:

$$\mathrm{DC} = \mathrm{CRM} + \mathrm{CL} + \mathrm{CU} \tag{14.8}$$

Johnston and Penninger (1989) suggested using Eq. (14.9) in an industrial unit which is designed based on a CO_2 laboratory or pilot unit data:

$$\left(\frac{f_{\mathrm{CO_2}}}{V_{E\rho_B}} \right)_2 = \left(\frac{f_{\mathrm{CO_2}}}{V_{E\rho_B}} \right)_1 \frac{H_{B1}}{H_{B2}} \tag{14.9}$$

where H_B represents the bed height (m) and ρ_B the bed density (kg/m^3).

Indirect costs. The indirect costs can be expressed as percentages of the fixed capital investment. It usually varies from (20 to 40) % of the equipment cost. A typical indirect cost distribution can be: Insurance 1 % FCI, Depreciation 10 % FCI, Maintenance 2 % FCI, Property tax 1 % FCI (Alvarez et al. 2009). Other general costs including marketing, research and development as well as administrative costs can represent about 5 % of the total investment (Peters and Timmerhaus 1991).

Perrut (2000) reported that prices (represented by a dimensionless price index PI on a logarithmic scale) are near to a straight line with a slope of 0.24 versus the log of the product of the total volume V_T and the solvent flow rate Q:

$$PI = A(10\, V_T Q)^{0.24} \tag{14.10}$$

The solvent flow rate Q is proportional to the total extractors (+column) volume V_T, indicating that the cost of a unit approximately increases as the square root of the capacity. This correlation is applicable to a large range of capacities, from the bench scale (0.5-L autoclave) to the industrial scale (500-L autoclave); however, it underestimates prices of the small bench-scale equipment (0.2-L autoclave) and overestimates the price of much larger units, like those for hops or coffee/tea processing (Perrut 2000).

14.8.3 Manufacturing Cost of Extracts Obtained by HPP

The production cycle comprises the manufacture and the sale. Both activities generate several expenses that can be grouped in *direct manufacturing costs*. The latter include items that can be directly related to the product fabrication such as general utilities (heating, cooling, pumping), raw materials, operation labor, plant maintenance, research and laboratory work, depreciation, taxes and insurance, as well as the *general costs* associated with the selling and administration activities. The sum of both constitutes the *total production cost* (Alvarez et al. 2009).

According to Rosa and Meireles (2005), the *manufacturing* cost (COM) can be determined by the sum of the *direct cost*, *fixed cost*, *and general expenses*. The direct costs are dependent on the production rate while costs such as territorial taxes, insurances, depreciation, etc., are not dependent on the production rate and are known as fixed costs. General expenses are associated with business maintenance and include administrative, sales, research and development, among others.

An estimate of the COM of extracts obtained by HPP can be obtained applying the methodology presented by Turton et al. (1993). The authors suggested to estimate COM in terms of five main costs - *raw material*, *operational labor*, *utilities*, *waste treatment*, *and investment* – according to:

$$\mathrm{COM} = 0.304\,\mathrm{FCI} + 2.73\,\mathrm{COL} + 1.23 \times (\mathrm{CUT} + \mathrm{CWT} + \mathrm{CRM}) \qquad (14.11)$$

where COM (USD/kg extract) is the manufacturing cost, FCI (USD/kg extract) is the fraction of investment, COL (USD/kg extract) is the operational labor cost, CUT (USD/kg extract) is the utility cost, CWT (USD/Kg extract) is the waste treatment cost, and CRM (USD/kg extract)is the raw material cost.

In Eq. (14.11) the fraction of the investment on a year basis is given by the product of the total investment and the depreciation rate. Another part of the investment is the initial amount of carbon dioxide needed to fill the CO_2 reservoir. In general, this cost is negligible if compared to the extraction unit cost. The total operational labor is expressed in terms of man-hour per operation-hour.

The utility is estimated considering the energy involved in the solvent cycle using the pure CO_2 temperature–entropy diagram, as suggested by Brunner (1994, 2005). The values of specific enthalpies can be obtained from this diagram using the pressure and temperature for each part of the process.

In order to estimate the COM it is important to have knowledge of aspects regarding mass transfer and equilibrium as indicated before, namely extraction time and the yield of extract obtained during that time. It is generally considered that the industrial scale supercritical extraction unit should have the same performance as that of a laboratorial scale unit, if the particle size, bed density (mass of particles per unit of column volume), and the ratio between the mass of solid and the CO_2 flow rate are kept constant. This assumption should be precise if the scale-up is done by increasing the column diameter and the CO_2 is distributed similarly.

14.9 Results of Estimation Analysis for the Industrial Application of HPP

As indicated previously, some of the reasons for the limited implementation of HPP in the food, nutraceutical and pharmaceutical industries are related to the difficulties to perform economic evaluations, the relatively high capital investment and the consideration of HPP products like simple substitutes of conventional market references.

Table 14.5 summarizes the results obtained in the literature for the COM estimation of HPP. It can be seen that though high pressure technological applications have been known for over 40 years now, estimations of the costs of their industrial applications are very recent, most of them in the last decade. In addition, they are generally applied to natural extracts in batch-type apparatus. The estimation of costs is generally performed using empirical correlations like Eq. (14.11), the application of which can lead to under- or overestimation of specific items. At this point the calculation of the equipment cost and the capital investment is crucial; it is thus recommended to avoid generic correlations based on specific design parameters which have to be updated, and to obtain the information directly from a supplier. The accuracy of the results will depend on the precision of the correlations and the assumptions considered. Better estimations, close to the industrial implementation, correspond to a business plan strategy, and should involve all the process previously described, with a complete financial plan that can include cost of marketing, packing, labeling, monthly prediction of accounting, as the same as quantitative financial ratios and sensitivity test.

In general, the results are highly depending on the FCI, reducing approximately to a third their manufacturing cost when increasing the extractor's volume an order of magnitude (Albuquerque and Meireles 2012; Prado et al. 2010). Even though considered as substitutes, several processes are industrially viable due to the fact that the COM is lower than the market price of the corresponding product, e.g. peach almond, spearmint, grape seed, clove bud or sunflower (Mezzomo et al. 2011). These good results are obtained for high productions in extractors of about one cubic meter volume. As indicated previously, the price reduction for high scale has been the only strategic criterion for actual implementation of industrial HPP plants. However, for low-prices niche products, high equipment costs and high productions introduce a risk factor regarding competitors, market price fluctuations or even changes in the interest rate that can affect the amortization (Perrut 2000). Thus, optimization of design in terms of COM and test of sensitivity give a real impression of the riskiness of the project. As an example, not optimized operation conditions can affect significantly equipment cost (FCI) and hence increase the COM by 30 % (Melo et al. 2014), or a 5 % increase of the interest rate can increase the pay back of the project (Fernández-Ronco et al. 2013b). The raw material costs vary from values close to zero in by-products to high expenses that are affecting more COM that FCI, specially for cases where transport, heating, drying, or milling

Table 14.5 Literature values for COM estimations of HPP processes

Extract	Tipe	Vol (L)	Production (t/years)	COM (USD/kg)	Market (USD/kg)	Reference
Annato seeds	BS	10–1,000		109.27–300		Albuqueque (2012)
Anacardium leaves	B	10–1,000		5.91–9.45		Leitao (2013)
Butiri oil	BS	800		25–100	15	Prado (2010)
Capsicum oleorresin	CF	18	10	36–50		Fernández-Ronco et al. (2013b)
Clove bud	BS	800		9.15	40	Rosa and Meireles (2005))
Ginger	BS	800		99.8	100	Rosa and Meireles (2005)
Grape seed	B	0.5–1,000		133.16–429	230	Farías-Campomanes et al. (2013)
Grape seed	BCC	2400	3,000	5.9	10–30	Fiori (2010)
Mango leaves	BS	30–3,000		92–900		Prado (2010)
Marigold	B	800–900		611–824	283–584	Mezzomo et al. (2011)
Palm Fiber	BS	800		35	1.74	Prado (2010)
Peach almond	B	800–900		5–25	40–150	Mezzomo et al. (2011)
Pink Shrimp	B	9,000	1,600–12,000	17.78–71.65	15–60	Mezzomo et al. 2013
Pupunha	BS	800		20		Prado et al. (2010)
Spearmin	B	800–900		242–913	574–1,650	Mezzomo et al. (2011)
Spent coffee grounds	B	3,000	454	5–15		Melo et al. (2014)
Stripped weakfish	B	1,000		4		Aguiar
Sunflower	BC	5,000		0.7	5	Bravi et al. (2002)

B batch, *BSC* batch semicontinuous, *BC* batch continuous, *BCC* batch countercurrent continuous, *CF* column fractionation

steps must be included (Leitao et al. 2013; Rosa and Meireles 2005; Mezzomo et al. 2011).

In marketing strategies focused on nutraceuticals or "gourmet" products, low productions are preferred and selling prices increase rapidly due to the application of products for health care or just because of their exclusivity (Shariaty-Niassar et al. 2009; Hughes 2009). In these cases the economic viability of the process has been also demonstrated (Fernández-Ronco et al. 2013b), but the industrial application is still difficult.

14.10 Costs of Industrial Applications

Irregardless of the difficulties outlined above, there are several products obtained by HPP available in the market today. They are usually natural extracts produced in semicontinuous batch-type apparatus for high volume of processing (King et al. 2011; Perrut 2000). As indicated previously, there are risks for the introduction of high volume production of extracts not widely accepted by the market; thus a novel idea of implementing versatile multipurpose plants is gaining ground. However, for such cases, special attention must be paid to cleaning and start-up steps, which can negatively affect the profitability (Perrut 2000).

Table 14.6 summarizes some customer prices of the available products in the market obtained by HPP, where the range of values indicates the price interval for different quantity (high to low) orders. In Table 14.6 lower prices correspond to lower quality oils or extracts, while higher prices correspond to nutraceutical or pharmaceutical applications. Thus, for the pomegranate extract, prices accepted by the market ranges from (1–30) USD/kg for food applications, to (25–80) USD/kg for high quality food, to (50–200) USD/kg for nutraceutical applications and (200–300) USD/kg for those extract with anti-wrinkle properties. Sea Buckhorn seed extract prices vary from (2–100) USD/kg for food to (130–150) USD/kg for cosmetic applications. This evidence indicates that the market accepts high prices and the "high niche" strategy is possible in HPP.

For industrial multipurpose plants, the price for hop extraction varies between (1.5–2) €/kg for batch sizes of some 1,000 t (whole plant) depending on the hop variety. The price for the tea decaffeination varies between (3 and 5) €/kg raw material for batch sizes of some 10 t depending on the tea variety and the caffeine

Table 14.6 Prices for industrially available products obtained by high pressure processing

Extract	Market price (USD/kg)
Amaranthus caudatus	120–150
Black pepper	12–30
Borage seed	50–60
Clove	30–50
Crude palm	23–56
Evening primrose	45–55
Garlic	13–36
Goji seed	200–230
Jojoba	32–91
Nitraria seed	1–50
Peony seed	1–50
Pomegranate	1–30, 25–80, 50–200, 200–300
Safflower	1–50
Saw palmetto	190–200
Sea buckthorn seed	2–100, 130–150

Source: www.alibaba.com

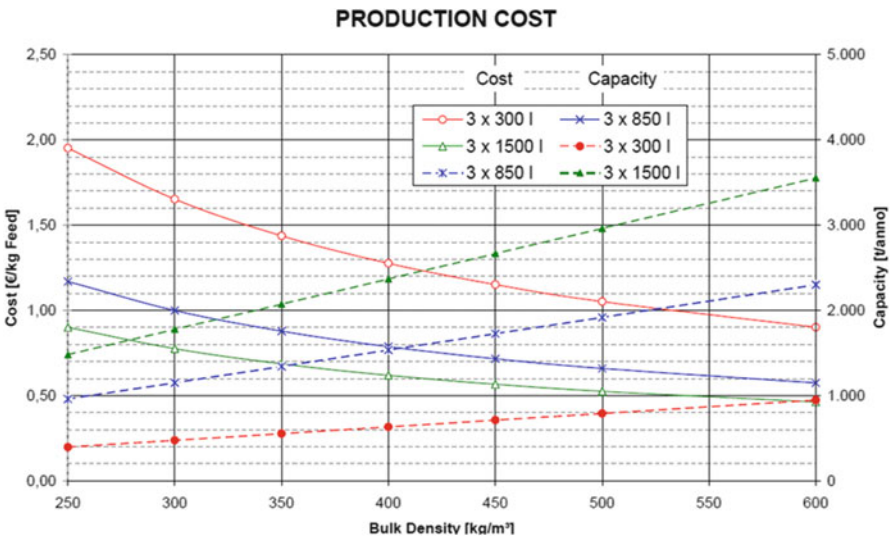

Fig. 14.12 Production cost of industrial multipurpose plants as a function of bulk density (Seidlitz et al. 2013)

content. The price for the extraction of specialties in small batch sizes (<1 t) is between (10 and 20) €/kg. In general, prices depend significantly on batch sizes and the optimum is achieved when one plant can be filled with only one product for the whole year (www.nateco.com).

The production cost of industrial plants is highly dependent on the extraction volume, the production rate, and the characteristics of the raw material, like bulk density (Fig. 14.12). The use of high volumes reduces cost in a ratio similar to those observed in the previous COM estimation section. On a Kg extract basis, and assuming very restrictive conditions, i.e., a 10 % extraction yield and minimum bulk density, productions costs vary from 9 €/kg for 3 × 1,500 L extraction, to 12€/kg and 20 €/kg for 3 × 850 and 3 × 300 L extractors, respectively (Seidlitz et al. 2013).

Summary and Conclusions

In spite of the lack of implementation of high pressure processing in the food, nutraceutical and pharmacy market, high pressure technology is ready to be widely used for the development of new products. Nowadays several processes are profitable, and HPP products with proven therapeutic characteristics are available. This chapter presets some information for researchers to be able to understand the basic concepts of the economy related to the industrial implementation of HHP. Based on a Business Plan strategy, several tools and strategies are presented so that the real possibilities for HPP products to be

(continued)

(continued)

scaled up to industrial plant and for the introduction of the respective products in the market can be determined.

The correct strategy seems to indicate that the market niche of HPP products should be changed from simple substitutes, to a "top level", "high quality" or "neutraceutical" grade. Several emerging opportunities for regulations, labeling, market demands and marketing strategies, have to be exploited. If scientists can be involved in the different steps for the industrial development of HPP products, their future can be very promising and profitable.

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