Irena Baranowska *Editor*

Handbook of Trace Analysis

Fundamentals and Applications

Springer

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Editor

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Fundamentals and Applications

With contributions by

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Part I
General Issues

Chapter 1
Characteristics and Specificity of Trace Analysis

Adam Hulanicki

1.1 Introduction

The concept of traces in chemistry probably originates from descriptions of the purity of chemical reagents, which in the nineteenth century included a statement such as "trace" for minor components when a more accurate description was not needed or not possible. One early example of determination of traces was the Marsh test, developed as early as 1836 for the determination of very small amounts of arsenic, which was of primary importance in toxicology.

The term "trace analysis" (in German *Spurenanalyse*, in French *analyse de traces*) was introduced into analytical chemistry in the twentieth century, when it was stated that very small amounts of admixtures can seriously influence the properties of many technical materials (e.g., metals and semiconductors). Significant developments came rapidly in the 1940s and 1950s with the advent of nuclear technologies, which required the determination of minute amounts of components and impurities of less than 10^{-3}–10^{-5} %. Further progress in many new fields of human activity (e.g., electronics and semiconductors) required continuous development of analytical technology. Other aspects of human activity also demanded analytical determination of very low concentrations of elements and compounds, particularly biologically active components. This was important in such fields as medicine, biology, biochemistry, and environmental science. In these areas, final conclusions are based on qualitative and quantitative analytical data concerning the elemental and/or molecular composition of studied objects at very low levels, even down to concentrations of 10^{-8} % [1–6].

Responsible analytical procedures obviously need to use the corresponding quality of reagents. In most cases, in analysis it was not possible to use "technical"--

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grade reagents with content of the main component in the 90–95 % range. Even "pure" reagents, with 99.0–99.9 % of content comprising the main component, were not acceptable. Therefore, grades of reagents were introduced that were described as "pure for analysis," containing at least 99.9–99.99 % of the main component; "chemically pure," of 99.99–99.999 % purity; and "spectrally pure," of 99.999–99.9999 % purity. Reagents of "nuclear purity" contained over 99.9999 % of the main component. These terms indicate the progress made in the analytical possibilities for determination of very small concentrations of species.

Knowledge of the role of many elements in the processes occurring in living organisms has stimulated development of analytical procedures applicable for determination of very low concentrations of elements in biological objects, both of animal and plant origin. The term "microelement" became commonly used, not only because of their low concentrations but also because of their special role in many natural systems. The need for procedures for their determination caused the development and publication of numerous studies devoted to trace analysis at the end of the nineteenth and beginning of the twentieth century.

Progress in the analytical chemistry of extremely low concentrations of analytes promoted organization of many scientific conferences of a general character, as well as some devoted to special aspects of trace analysis. Several special monographs also have been published [1–6]. It should also be noted that the term "trace analysis" has often changed its meaning. In the 1930s it described concentrations of the order of 10^{-2} %. Two decades later, that limit shifted to 10^{-5} %. Soon after, analysts introduced the concepts of microtraces (10^{-4}–10^{-6} %), ultramicrotraces (10^{-7}–10^{-9} %), and even submicrotraces (10^{-10}–10^{-12} %). However, such subdivision of the trace range is rather arbitrary because all concentrations below 10^{-2} % are often included in the trace range.

In contemporary trace analysis of complex objects, initial steps are usually necessary for separation and preconcentration of an analyte. Often, just those two steps allow proper determination; however, it must be remembered that each step can introduce errors. The errors can be either positive (e.g., by accidentally introducing impurities) or negative because of loss of analyte [4, 5]. In inorganic trace analysis, the analyte (element) is not convertible during the working procedure so there are practically no limits to the use of drastic techniques. The number of possible analytes is restricted to the number of known elements; however, various analytical procedures have been developed for each element that depend on the character and composition of the studied object. In organic trace analysis, the number of analytes is theoretically unlimited and can increase continuously. Obviously, knowledge of the origin of the samples gives analytically useful information; nevertheless, the analyst should always be aware that some unpredicted species could disturb the analytical process.

An important aspect of organic analysis is the instability of many compounds in the changing conditions of the analytical process. This could restrict the use of more drastic procedures in the course of determination. Transformation of organic species can occur as a result of chemical reactions such as hydrolysis, oxidation, reduction, and enzymatic or microbiological processes, as well as under the

influence of physical factors such as temperature and radiation. This demands broad knowledge of the properties of the system, even in the case of determination of inorganic traces. Sometimes it is helpful to make a kind of mental extrapolation of the analytical result back to the initial stage, as well as a critical evaluation of all steps of each analytical operation, even processes that might occur during storage of the sample.

The problems increase when the analyzed object contains other species with chemical properties similar to those of the analyte. Often there are no specific procedures for a given compound, and the analytical process should start with separation procedures. Among these are, in the first line, various useful chromatographic procedures, which constitute a very important component of the whole analytical process. Therefore, the rapid progress in organic trace analysis in the second half of the twentieth century was connected with the development of chromatography and physical methods for species identification.

A relatively simple case is when one well-known compound is determined in a given product. Examples are determination of an antioxidant, vitamin, or colorant in a food product or of a plant protection compound in a specified agricultural object. The situation becomes complicated when there is the probability of chemical transformation of the analyte in the sample, as happens frequently during determination of a drug, toxin, or narcotic in urine, blood, or other biological object. In such cases, it is necessary to take into account probable biochemical processes occurring in a given medium, with formation of initially unsuspected metabolites with a priori unknown composition.

Determination of traces of organic compounds has become especially important, as many of those species have been introduced into the environment in an uncontrolled way. Sometimes those substances were used because of their special positive activity, but harmful effects became obvious later. A known example is the insecticide DDT (dichlorodiphenyltrichloroethane), which does not decompose rapidly in nature but accumulates dangerously in many organisms. Among other active species formed in many industrial processes are polycyclic hydrocarbons and dioxins (chlorinated derivatives of dibenzodioxins), both highly toxic species that are now present in nearly all environmental media. Determination of individual species is difficult because they are accompanied by numerous compounds of similar structure (congeners, isomers, homologs) and closely related chemical properties.

These comments indicate the difficulty of the task of organic trace analysis. It is also necessary to mention that confirmation of the quality of the determination is more difficult than for trace inorganic analysis (where certified reference materials can be used), because of the greater variety of analytes and their chemical instability.

A case that joins the problems of trace organic and inorganic analysis is speciation analysis, whose importance has been appreciated in the last few decades. Speciation studies became the center of interest of inorganic analysts when they demonstrated that various derivatives of the same element can exert fully different chemical and biological activities. Speciation analysis requires determination, at the trace level, of organic compounds of metals and nonmetals in various environmental objects. These compounds can be fully of anthropogenic origin

(e.g., organic tin derivatives) or formed as a result of interaction of elements and their inorganic derivatives with naturally occurring organic species. The need for their determination led to development of so-called hyphenated techniques (or coupled techniques). These procedures combine elements of separation techniques (e.g., gas chromatography) with very sensitive instrumental techniques (e.g., mass spectrometry). Such procedures, due to their excellent sensitivity, made it possible to discover new species occurring in trace amounts [6].

Correct results in trace analysis are not only connected to a change in the scale of determination or use of a more sensitive detector in the final step of a multistep procedure. These aspects must obviously be taken into account, but they do not decide the success of trace analysis. Each step, and practically each function, could introduce a given uncertainty in the final result. Thus, the final result is connected with much larger uncertainty than the result of a simple measurement. The analyst must be aware that the smaller the concentration of analyte, the greater the uncertainty of the final result. This is usually a result of the following factors:

- Large excess of other components in the sample
- Use of multistep analytical procedures
- Possibility of positive errors because of contamination of the studied object
- Possibility of loss of analyte, for example as a result of adsorption or degradation
- Interference caused by the presence of other components
- Low precision of instrumental measurements compared with chemical measurements for determination of large amounts (high concentrations)

Detailed analysis of results obtained in many laboratories has indicated that precision generally decreases when the concentration of analyte decreases. This dependence is formulated as the Horwitz diagram [7] (Fig. 1.1). The diagram

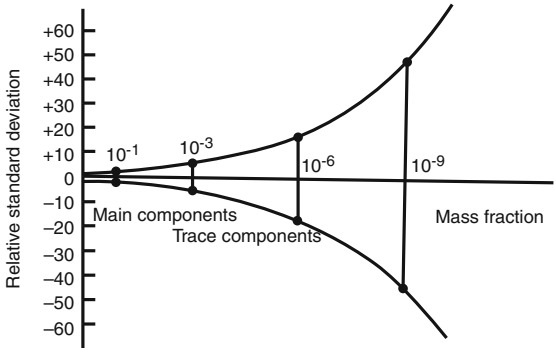

Fig. 1.1 Horwitz diagram showing dependence of standard deviation (%) on analyte content [7]

indicates that the uncertainty of the final result is two times larger for concentrations two orders smaller. The statement refers only to precision of the result, but says nothing about its accuracy. It should be stressed that even a coefficient of variation of the order of 20 % at an analyte concentration of 10^{-4}–10^{-6} % does not disqualify the result. On contrary, a value smaller than a few percent suggests error in calculation or a tendency to unfair improvement of the quality of results.

1.2 Units of Content Used in Trace Analysis

In trace analysis, the units used for component content should be appropriate for the amount. The most proper system is to give the mass fraction (w_B) of the trace component (B) in SI units with the appropriate prefix: milli- (10^{-3}), micro- (10^{-6}), nano- (10^{-9}), pico- (10^{-12}), femto- (10^{-15}), or atto- (10^{-18}) (Table 1.1). The component content is relative to the mass of the whole sample expressed in grams or kilograms. A short derivative system is often used that gives the number of parts of the trace component for a million (ppm) or billion (ppb) parts of the sample. This system is commonly used; however, one should be aware that term "billion" in American nomenclature denotes 1,000,000,000, which in the European terminology means milliard, whereas "European billion" is 1000 times larger and corresponds to "American trillion." The short description ppb (parts per billion) might therefore be understood differently. A similar error could also appear for parts per quadrillion (ppq). The International Union of Pure and Applied Chemistry (IUPAC) has finally accepted the American system, such that 1 ppb means $1/10^9$. Such short description of units also brings the danger that "ppt" might sometimes be incorrectly read as parts per thousand [8].

Some confusion may also arise when one does not remember that these expressions strictly refer to mass rather than volume units. The difference is negligible when only aqueous solutions are considered because 1 L is practically equal 1 kg, but it should not be forgotten when other liquid systems are considered. In such cases, mass concentration units (c_B or γ_B) should be used, being the ratio of analyte mass to the volume of liquid sample. Such an approach is particularly incorrect in the case of solid samples when their density differs significantly from unity, and

Table 1.1 Units of content used in trace analysis

Mass fraction (w)	Percentage content (%)	Mass ratio		Mass concentration	Non-recommended units
10^{-3}	10^{-1}	1 mg/g	1 g/kg	1 g/L	
10^{-6}	10^{-4}	1 µg/g	1 mg/kg	1 mg/L	1 ppm
10^{-9}	10^{-7}	1 ng/g	1 µg/kg	1 µg/L	1 ppb
10^{-12}	10^{-10}	1 pg/g	1 ng/kg	1 ng/L	1 ppt
10^{-15}	10^{-13}	1 fm/g	1 pg/kg	1 pg/L	1 ppq
10^{-18}	10^{-16}	1 ag/g	1 fg/kg	1 fg/L	

when there is no clear statement about whether the analyte mass refers to the mass of the whole sample or to the volume of the sample.

Sometimes, particularly when the detectability of various methods is being compared, the units of mass fraction are expressed in the logarithmic scale. Thus, instead of mass fractions 0.000001 or 0.000005, their negative logarithms, 6.00 or 5.30 respectively, are given. This is particularly convenient when the analytical signal is a logarithmic function of concentration, as occurs in potentiometric measurements.

In the case of evaluation of the detection limit of gaseous impurities in gases, particularly in air, the amount of the detected or determined substances is usually given in volumetric units such as milliliters per cubic meter (mL/m^3), which is equivalent to ppm (volumetric). Presentation of the content of gaseous components in mass units (mg/m^3) is less convenient, because it depends on temperature and pressure and should be given under standard conditions (e.g., 20 °C, 1013 mbar).

As the main aim of trace analysis is usually determination of the mass (expressed as the number of moles) of a given component in a studied sample, molar concentration is generally not used. Some exceptions are electrochemical methods, where the analytical signal (e.g., current intensity) is a direct function of molar concentration [9]. Therefore, in voltamperometric techniques the detection limits are usually given in molar concentration units (Table 1.2). Thus, for nickel (molar mass $M = 58.7$ g/mol) the detection limit in inverse voltammetry is approximately 6×10^{-9} mol/L, and is expressed as a mass fraction, 3.5×10^{-7} g/dm^3, or as a percentage, 0.35×10^{-7} %. In spectrophotometry, when concentrations are given in molar units then molar absorptivities are also used. For example, molar absorptivity $\varepsilon = 5 \times 10^{-4}$ L/mol cm corresponds (for molar mass $M = 58.7$ g/mol) to molar absorptivity $a = 5 \times 10^4/(58.7 \times 10^3)$ mL/g cm (i.e., 0.85 mL/g cm).

Table 1.2 Detectability of some electrochemical techniques [10]

Technique	Detectability	
	mol/L	g/mL
Constant current polarography (DME)	10^{-5}	5×10^{-7}
Pulse polarography (DME)	5×10^{-7}	2.5×10^{-8}
Differential pulse polarography (DME)	10^{-8}	5×10^{-10}
Square wave polarography (DME)	10^{-8}	5×10^{-10}
Inverse voltamperometry (HMDE)	10^{-10}	5×10^{-12}

Molar mass was arbitrarily assumed to be 50 g/mol
DME dropping mercury electrode, *HMDE* hanging mercury drop electrode

1.3 Trace Analysis in Development of Methods of Chemical Analysis

Development of the techniques and methods of analytical chemistry gave more efficient possibilities for trace determination in analytical samples. In the first half of the twentieth century, the chemical procedures had limited detectability. For determination of small (trace) amounts, relatively large samples needed to be used. Both mass and volume procedures were not convenient, and real progress was made by the introduction of emission spectrometry and spectrophotometry based on formation of sensitive spectrophotometric reagents.

Emission spectroscopy with constant current arc excitation was made possible in the 1950s, allowing determination of contents of 10^{-3} % and, in special cases, even 10^{-6} % in solid samples. Such determinations could be described generally as semiquantitative; however, in studies of metals and mineral objects they were of great importance. Similar limits were obtained with the use of flame photometry for alkali and alkaline elements in liquid objects.

Spectrophotometric methods (photometric or colorimetric) with application of color-forming reagents made possible the determination of many metallic elements at the 10^{-4}–10^{-5} % level. As the equipment for such procedures was easily accessible, and the analyzed sample was relatively easily soluble, these procedures were commonly used [11, 12]. Because there was knowledge of the physicochemical chemistry of those procedures, based on specially synthesized organic reagents, they gained popularity in the 1960s. Nevertheless, the limited magnitude of molar absorbance did not allow significant progress in detectability.

Electrochemical procedures, and primarily polarography in its classical version, made it possible to determine ions in solution at the level of 10^{-5} mol/L, which corresponds (for $M = 60$ g/mol) to a concentration of 6×10^{-4} g/L (or 6×10^{-5} %). Taking into account the need to dissolve solid samples, with a dilution factor of at least 10, it was not possible to consider classical polarography as a procedure adequate for determination of traces, according to criteria developed in the middle of the twentieth century. By contrast, inverse (stripping) polarography, based on preconcentration in situ, allowed determination of amounts that were three or even four orders of magnitude smaller [9, 10]. Contemporary polarographic methods (pulse, square wave) enable determination of concentrations significantly lower than in classical polarography, not only for inorganic species. Thus, even without preliminary preconcentration, electrochemical procedures have been successfully applied in trace analysis, in the absence of interferents.

The methods of atomic spectroscopy, important in the first decades of the twentieth century, again played a significant role in the 1960s as a result of new inventions (Table 1.3). Development of atomic absorption, in particular with graphite furnace atomization, resulted in new procedures that could measure down to the 10^{-7} % level. Somewhat lower concentrations could be determined with generation of volatile compounds, in particular hydrides; however, this was restricted to only a few elements. The development of atomization and excitation in

Table 1.3 Approximate detection limits of elements in selected spectrometric methods

Detection limit (ng/g)	FAAS	ICP-OES	GF-AAS	ICP-MS
100	Ag, Se, Al, Sb, V, Pb	K, Pt, Ce, Pt, Pd, Se, Na, Pb, Sb, Sn, Al. As, Ni, Au, W	Hg	
10	Ba, Co, Au, Ni, Fe, Cr, Mn, Ca, Cu	Co, Ni, Cr, Ag, V, B, Cu, Fe, Cd, Zn, Li, Ti	Se, Li, As, Pt, Sn, V	K, Ca
1	Ag, Zn, Cd, Li	Be, Bi, Sr, Ca, Mn, Ba	Sb, Au, Bi, Ni, Ca, Pb, Ba	Fe, Mg, P, Br, Se, Zn, Cr
0.1	Mg	Mg	Co, Fe, Cu, Al, Mn, Cr, Ag, Mg, Cd	B, Na, Hg, Co, Mn, Li, Be, Au, Pb
0.01			Zn	Cd, Mg, Al, Ni, As, Hg, Ag, Cu, Pd, Ba, Mn, Pt
0.001				Bi, La, Ce, In

In the case of GF AAS, sample volume was assumed to be 20 μL
FAAS atomic absorption spectrometry with flame atomization, *ICP-OES* atomic emission spectrometry with inductively coupled plasma excitation, *GF-AAS* atomic absorption spectrometry with graphite furnace atomization, *ICP-MS* mass spectrometry with inductively coupled plasma ionization

inductively coupled plasma has not shifted the limit of detection below 10^{-7}–10^{-8} % but has significantly broadened the range of analyzed metallic and non-metallic elements. Reaching the 10^{-9} % limit was possible only with inductively excited plasma as ion source and with mass spectroscopic detectors [2, 13]. Progress in construction of instruments and new concepts in ionization methods shifted the limit of detection to 10^{-10} % for some inorganic and organic analytes.

In molecular as well as atomic spectroscopy, significant improvement in detection can be obtained by using laser-excited fluorescence. In atomic spectroscopy, electrothermal atomization with laser-excited fluorescence can improve the detection limit by two orders of magnitude. In molecular spectrofluorimetry, the excited items are mainly metal complexes with organic ligands. Such effects are a result of the increase in laser strength and decrease in the background; however, this can be observed for only a limited number of analytes [14]. The improved specificity and sensitivity of fluorescence of chemoluminescent reagents and chromatographic detectors has resulted in the lowest limits of detection, of the order of 10^{-12} % [15]. Neutron activation analysis enables determination of some elements in solid objects, without their dissolution, over a very broad range of concentrations at the level of 10^{-8}–10^{-9} % [16].

There are only a few analytical techniques that allow analysis of samples without chemical pretreatment. In most practical cases, the spectroscopic and electrochemical procedures require initial dissolution of the object. Such treatment

introduces the possibility of preconcentration of the analyte and, as a consequence, improved detectability. Nevertheless, such multistep procedures can often result in loss of determined elements as well as introduction of contaminants, resulting in analytical errors.

Determination of traces of organic compounds in organic objects presents special difficulties. The analytical procedure is usually composed of several steps and can include purification, separation, and preconcentration of the analyzed sample, followed by analyte determination. Significant progress in organic analysis was a result of development of chromatography and other separation techniques in application to organic species. New achievements in organic and biological chemistry stimulated rapid progress in procedures for quantitative determination of many compounds with initially unknown physicochemical properties. Gas chromatography offered determination of small amounts that were outside the possibilities of detection with classical chemical methods. In the 1960s, the insecticide DDT was determined by classical procedures at the microgram per gram (μg/g,10^{-4} %) level, whereas knowledge of its harmful role in the environment and biological objects was obtained only as a result of progress in gas chromatography and determination at concentrations five orders smaller.

Determination of traces of organic compounds needs better procedures if the final stage of determination is less selective. For example, in some cases, selective immunochemical procedures do not need complex procedures of separation and isolation of the analyte. When the detector is nonselective, the final determination can be made with contemporary chromatographic procedures. The great advantage of these techniques is the possibility of simultaneous determination of several analytes, even when they are of similar molecular structure. Nevertheless, removal of a large excess of apparently neutral species is often required. Such a case is determination of polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and other groups of compounds at the nanogram per gram (ng/g) level in environmental samples containing several tens of various, formally inert, species.

The variety of chemical and physical properties of determined trace components required rapid development of both gas and liquid chromatographic instrumentation. Many laboratories worked on the development and optimization of the structure and composition of stationary and mobile phases according to the properties, structure, and size of separated molecules. Studies were devoted to correlation of their physicochemical characteristics with chromatographic parameters. For typical applications, numerous commercial products and new instrument versions were developed.

For determination of minute quantities of analytes, several chromatographic detectors were developed. These were either selective for some groups of compounds or allowed simultaneous determination of various types of species. For example, the flame ionization detector is universal for organic compounds, but its detectability is of the order of 10^{-7} %. By contrast, the electron capture detector is selective only for halogen organic species but is two orders of magnitude more sensitive. Generally, fluorescence detectors possess excellent sensitivity. Progress in construction of mass spectrometers as detectors significantly widened the

possibilities of gas and liquid chromatography for determination of organic species down to concentration of 10^{-10} % (pg/mL).

In most cases, the use of chromatography in trace analysis needs preliminary preparation of analytical samples. For such purpose, beside classical liquid–liquid extraction, special systems were developed for extraction from or into the gas phase, or for extraction with the participation of solid phases. These systems enabled very low and very selective determination in the final step, even for microsamples.

Of the various analytical techniques, methods using mass spectrometry with isotope dilution play a special role. Initially they were used for determination of inorganic analytes, but later, in the last decades of twentieth century, they were also used for the analysis of organic species. These methods require the use of isotope-labeled compounds, but lead to very good precision and accuracy, as required in definitive procedures [14].

Progress in contemporary analytical chemistry is often connected with miniaturization of working scale; however, this does not generally mean improvement in detection limits. It is necessary to mention that electronic systems for automatic recognition of complex systems can provide qualitative information about the studied object at the 10^{-7} % level.

Rising interest in the chemistry of biological systems resulted in increasing application of biochemical techniques in trace analysis. A number of enzymatic and immunological reactions are characterized by high specificity and applicability for analytical determination at the picogram per milliliter (pg/mL) level. Progress in these techniques was stimulated by the need to determine organic species in a variety of samples and by legal requirements concerning maximal impurity content in a variety of environmental objects (water, soil, air), typical agrochemical samples, and food. Another important field of interest is monitoring of pharmaceutical residues in biological and environmental samples. Similar trace determinations are of importance in criminology and in the search for natural species of required activity. Those regions of interest continue to stimulate the search for new analytical procedures in a variety of objects.

1.4 Limits of Detection and Determination

Detectability is the basic parameter that provides information concerning the applicability of a given technique in trace analysis. The quantitative characteristic of detectability is the detection limit (DL, LOD, L_D), which defines the smallest amount, or concentration, of a given analyte that can be experimentally stated with a given probability. A numerical value of L_D has sense only when detailed experimental conditions for its evaluation are given. Sometimes the term “instrumental limit of detection” is used. It should refer to the particular specified instrumentation

used for its determination, usually on the basis of "pure" standard solutions of the analyte, in the absence of other substances that might directly affect the magnitude of the analytical signal. Such a limit of detection is often given in general textbooks for comparison of various techniques and in advertisement papers, mainly without any detailed description of the conditions used for its evaluation. Because detectability of various analytes (elements, compounds) using the same technique can differ significantly, such a characteristic often refers to supposedly optimal conditions. When a range of detection limits is given for a discussed method, it probably refers to data obtained in best and least favorable conditions. In some techniques (e.g., electrochemical), the detection limit only slightly depends on the nature of the analyte (Table 1.2).

In analytical practice, the concept of a detection limit for an analytical method is useful. It should be evaluated in conditions directly corresponding to real conditions for the whole analytical procedure and take into account real situations that the analyst meets in performing analysis. Such information should take into account all interferences that occur in the analytical process, including incompleteness of chemical and physical procedures (extraction, precipitation, distillation, etc.) and possible loss of analyte in the course of the whole analytical procedure. All these phenomena can directly influence the magnitude (and precision) of the final analytical signal.

Comparison of various analytical procedures used in trace analysis needs comparable conditions of determination and the same procedure for detection limit calculation. In some special cases the calculated limit of detection for "pure" solutions can differ by one order of magnitude in analysis of biological objects or food, by two orders in analysis of simple inorganic materials, or by as much as three orders for very complex objects such as mineral or geological items [13].

In experimental evaluation of the detection limit, each measurement carries an accidental error. When there are sufficient individual results it is assumed that the distribution of errors is normal (i.e., Gaussian), which for very small signals is not strictly fulfilled. Under such conditions the spread of experimental results is characterized by the standard deviation at the background level, S_B. Because exact determination of S_B might be difficult, it is generally assumed that it does not differ significantly from S_B close to limit of detection. Then, S_B can be calculated as follows:

$$S_B = \sqrt{\frac{\sum (\gamma_B - \overline{\gamma})^2}{n-1}} \tag{1.1}$$

where:

γ_B result of measurement
$\overline{\gamma}$ average value of n measurements

The limit of detection L_D is calculated as:

$$L_D = \overline{\gamma} + kS_B \tag{1.2}$$

In Eq. (1.2), k is a coefficient that depends on the assumed probability. Its value is usually $k = 3$. Note that when the results of measurements are given in the units of the analytical signal (e.g., number of counts, absorbance), then the obtained result should be divided by the sensitivity of the method, defined as the ratio of concentration to signal and corresponding to the slope of the analytical graph.

A similar situation occurs when the detection limit is evaluated on the basis of signals obtained for blind samples (in principle not containing the analyte), to which known amounts of the analyte, for example in the form of a standard solution, are added. The standard deviation of the crossing point of the extrapolated fragment of the calibration graph, $y = bC + a$, with the concentration (content) of the analyte is the basis for calculation of the detection limit of the procedure [17]. In such a case, error could be caused by the fact that the analyte added to the sample behaves differently to that present in real samples.

The evaluated limit of detection should not be given with more than two significant figures as it has only an approximate character, because the normal distribution of results may be doubtful in the range of very small concentrations. Even a very large number of measurements cannot guarantee that the result bears no serious errors.

In some situations, an apparent decrease in the detection limit can occur when the analytical procedure includes a preliminary preconcentration (e.g., by extraction or evaporation of a liquid sample). For example, if direct determination by atomic absorption spectrometry allows detection of 0.1 ng/mL metal in solution, then an increase in concentration after separation/preconcentration in the ratio 1:20 lowers the detection limit for the whole procedure to 0.005 ng/mL. Both values are useful and have real meaning when they are correctly described.

Comparison of detection limits of different analytical techniques for a given analyte is sometimes inadvisable. In some techniques (e.g., flame atomic absorption spectrometry or polarography), the magnitude of the signal does not depend on the amount of solution introduced to the analyzer. In other techniques the situation is different. For example, in atomic spectrometry with atomization in a graphite furnace, detectability depends on the amount of introduced sample. In such cases it is necessary to describe precisely all conditions of determination, in particular when there is no linear correlation between the amount of sample and the magnitude of analytical signal. Then it is possible to describe detectability by the absolute amount of analyte producing the signal; this, with a given probability, can be distinguished from the background level. Then, for example, we can write that the detection limit 1 μg/L for a 20 μL volume of sample corresponds to 20 pg of analyte.

In some techniques the signal is recorded as a function of sample residence in the detector. This is the case for chromatographic detectors, for which the value of the signal and, in consequence, the detectability depend on the time of residence and rate of registration. In such cases, the sensitivity of the detector is given in units that

are the product of mass and time. Thus, for example, detectability in gas chromatography [13, 18] is 10^{-11} g/s for a flame photometric detector, 10^{-12} g/s for a flame ionization detector, and 10^{-13} g/s for a thermo-ionization detector. Knowing all conditions of determination, those values can be recalculated for mass units or analyte content in a given sample. In practice, the limit of detection is assumed to be the smallest amount of substance that gives a signal twice the detector noise. The amplitude can be expressed as voltage, current intensity, or absorbance units and includes all accidental changes in signal that are larger than one per second.

The character of the analytical signal and its correlation with analyte content also influence the description of detectability. An example is measurement with ion-selective electrodes. The voltage (E) of the indicator electrode (and in fact the electromotive force of the cell) is, over a broad range, the logarithmic function of the activity of the determinant (c), which means that a graph can be plotted using coordinates E versus $\log c$. For activities (i.e., concentrations at constant ionic strength) below the detection limit, the analytical diagram is a straight line parallel to the concentration axis. The detection limit is usually accepted as the abscissa of the crossing point of the two linear parts of the calibration curve (Fig. 1.2). The reliability of such a detection limit depends on the standard deviation of points taken for plotting the analytical diagram.

Another situation appears in methods used for surface analysis, where detectability is given as a percentage relative to the monoatomic layer.

The numerical value of the detection limit is only an estimated magnitude with a given probability; nevertheless, how it was calculated should always be mentioned. Such a detection limit refers to a particular element or compound. It is necessary to be aware that the detection limit for a given analyte can differ from the result obtained by another analyst or using other instrumentation. A parameter closely related to detectability is the limit of quantification (LOQ, LQ). The term describes the smallest concentration (amount, content) that can be determined with

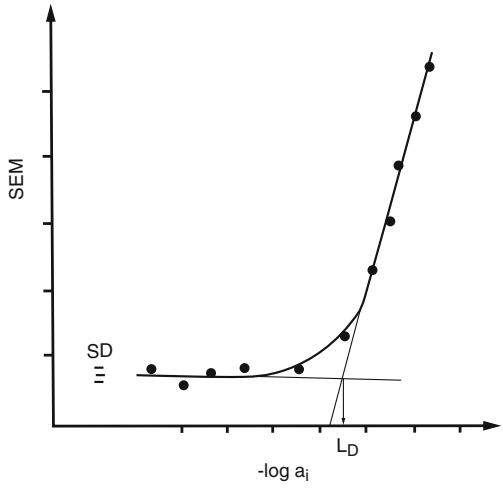

Fig. 1.2 Graphical evaluation of determination limit (L_D) in ion selective potentiometry (SEM - electromotive force)

"acceptable precision." The term "acceptable precision" means that it can vary depending on the given task. Frequently, it is assumed that the limit of quantification is a small multiplicity of the limit of detection. It is often accepted that the limit of quantification should be three times as large as the standard deviation at the level close to the blank, whereas the limit of determination is ten times that value. It is assumed that the accepted relative precision is at the level of 10 % [18].

It can also happen that the analytical diagram in the range of small concentrations is not linear. In such a case, the beginning of the linear part is often accepted as the limit of determination . This is based on the assumption that, starting from that point, the precision is sufficient for quantitative determination.

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Chapter 2
Quality of Analytical Results: Classifiying Errors and Estimating Measurement Uncertainty

Piotr Konieczka

2.1 Introduction

The most important parameter of each analytical result is its reliability. An analytical result is not a constant value; each result has two properties, error and uncertainty. The sources of both these parameters have to be known and their values determined (estimated).

All analytical results are obtained by applying an appropriate measuring procedure. The need for reliable results requires application of reliable analytical procedures, from sampling to final determination.

Conclusions obtained on the basis of analytical results should reflect the real ("true") content (concentration) of analyte in the analyzed object (sample). For this, two basic conditions must be fulfilled:

- The collected sample composition should reflect the composition of the analyzed object (requirement of sample representativeness).
- A measurement result should reflect the true content of analyte in the analyzed sample (requirement of measurement reliability).

The main trend in the development of analytical chemistry is the determination of lower and lower concentrations of analyte in samples of an increasingly complex matrix (trace analysis). In the case of trace analysis, several problems arise from the following [1]:

- Decreasing analyte concentration
- Increasing complexity of the sample matrix composition
- Introduction of new notions associated with the application of metrology principles in analytics

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– Necessity of traceability documentation and uncertainty estimation as necessary parameters of an analytical result
– Globalization and the associated need to compare results from different laboratories

Analytical data are a specific type of information. This information is not usually obtained through analysis of the whole object, but is based on the analysis of appropriate samples. Measurement results must be reliable, which means that they must accurately (both truly and precisely) reflect the real content (amount) of analyte in a sample that is representative of the material object under study. This task is extremely difficult and complicated, which poses a great challenge for analysts and requires attention to be paid to the problem of quality assurance (QA) and quality control (QC) of the obtained results [2].

The term "reliable data" is closely related to data quality. It is the quality of a measurement, and its control and assurance make it possible to determine and prove the reliability of a measurement.

Different aspects of quality have specific meanings in an analysis. According VIM (*International Vocabulary of Metrology*) [3], quality is defined as the degree of realization of specific requirements (those included in a standard of the quality control system and its "own" accepted requirements).

Analytical quality is an agreement of the obtained results of the chemical analysis with the accepted assumptions [3]. The quality of information can be divided into several components:

– Quality of results
– Quality of the process
– Quality of the instruments
– Quality of work and organization

The result of a measurement is the product of the analyst's work. The quality of this product depends on the quality of the tools used (i.e., the analytical procedure and the laboratory work). The quality of the obtained measurement result depends on the measurement error and the estimated uncertainty values.

2.2 Measurement Errors

Error is defined as the difference between the expected (true) value and the value obtained as a result of the determination. Thus, error can be calculated as a measured quantity value minus a reference value [3]. Measurement error is the consequence of the accuracy (as trueness and precision) of the analytical procedure applied for obtaining the measured quantity:

Accuracy: closeness of agreement between a measured quantity value (as a single result) and reference quantity value [3].

Trueness: closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value [3].

Both of these parameters are closely connected with the estimation of errors. Depending on the type of error, their influence on measurements varies.

The value of a single measurement result may differ (and actually always differs) from the expected (real) value. The difference is a result of the occurrence of different errors. There are three basic types of errors:

- Gross errors
- Systematic errors (biases)
- Random errors

With regard to the manner of presenting a determination result, one can distinguish between absolute and relative errors:

- *Absolute error* (d_x): described by the dependence:

$$d_{x_i} = x_i - \mu_x \tag{2.1}$$

where:

x_i value of a measurement result
μ_x expected (true) value

- *Relative error* (e_x): described by the equation:

$$e_{x_i} = \frac{d_{x_i}}{\mu_x} \tag{2.2}$$

With regard to the source of errors, one can distinguish:

- Methodological errors
- Instrumental errors
- Human errors

The total error of a single measurement result can be divided into three components, as described by the following equation [2]:

$$d_{x_i} = x_i - \mu_x = \Delta x_{\text{sys}} + \Delta x_i + \delta x_i \tag{2.3}$$

where:

d_{x_i} total error of a measurement result
Δx_{sys} bias
Δx_i random error
δx_i gross error

For a measurement series (at least three parallel analyte determinations in the same sample), there is a high probability of detecting a result(s) with a gross error. Gross error is the result of the single influence of a cause acting temporarily and causing the measurement result to differ significantly from the mean value (outlier result deviated). It appears only in some measurements and it is a random variable. This error is the easiest to detect and, therefore, the easiest to eliminate.

There are many known ways of detecting results with gross errors. Each is applied in certain specific conditions [2]. After eliminating results with gross errors, the trueness of the obtained final determination (most often the mean value of the measurement series) is influenced by biases and/or random errors.

An analytical result (arithmetical mean of a series of parallel measurements) can only have a bias and random error according to the following dependence [2]:

$$d_{x_m} = x_m - \mu_x = \Delta x_{\text{sys}} + \Delta x_m \tag{2.4}$$

where:

d_{x_m} total error of a determination result (arithmetical mean of the series of measurements)
x_m mean value of the series of measurement results
Δx_m random error

Random error is an error resulting from typical fluctuations in the experimental field. The value decreases in the case of multiple designation of the same analyte in samples of the same material. It is not possible to calculate this error for a single result, nor to predict its value. In spite of their low value, these errors are the basis for calculating precision and are a component of the uncertainty of analytical results [2].

Systematic error is an error that, during multiple measurements performed under the same conditions, remains constant. Its value cannot be calculated without knowing the actual value or a value contractually accepted as real. Systematic errors, which should be small, determine the trueness of measurement. This type of error can be a parameter of a single measurement or of an analytical process, in which case it is known as "bias" [2].

The determination of bias is one way to determine the trueness of an analytical method. If the determined bias refers to an analytical method, then with a large number of measurements, the random error is negligibly small with relation to the bias (when $n \to \infty$, then $s \to 0$) (where s is standard deviation).

In this case, the following dependence is true [2]:

$$d_{x_{\text{met}}} = E(x_{\text{met}}) - \mu_x = \Delta x_{\text{sys}} \tag{2.5}$$

where:

$d_{x_{\text{met}}}$ total error of a determination result for the applied analytical method
$E(x_{\text{met}})$ expected value for a given analytical method

The occurrence of bias makes a given series of measurement (analytical method) results differ from the expected value by a constant value; hence, results are either overstated or understated. There are two types of bias:

- *Constant bias* (a_{sys}): value does not depend on analyte concentration levels
- *Variable bias* ($b_{sys}\mu_x$): value depends (most often linearly) on analyte concentration levels

Total bias can be described by the dependence:

$$\Delta x_{sys} = a_{sys} + b_{sys}\mu_x \tag{2.6}$$

Assuming that the value of a random error is negligibly small compared with the bias value, one can present the following dependence:

$$x_m = \mu_x + \Delta x_{sys} = \mu_x + a_{sys} + b_{sys} \;\; \mu_x = a_{sys} + \left(1 + b_{sys}\right)\mu_x \tag{2.7}$$

Schematically, the impact of individual types of errors on the final result of the measurement is shown in Fig. 2.1.

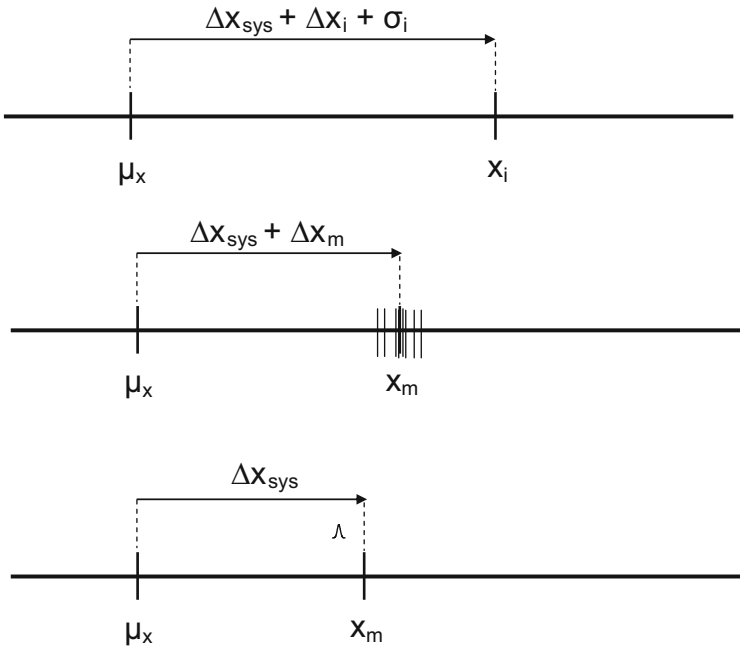

Fig. 2.1 The influence of the various types of errors on the final result of the measurement; Δx_{sys} bias, Δx_i random error for a single measurement, σ_i gross error, μ_x expected (true) value, x_i measurement result, x_m mean value of the series of measurement results, and Δx_m random error for a series of measurements

After rejecting results with a gross error and determining biases (regarding their values and correcting the determination result), the results still contain a random error. The value of the random error influences the precision of the obtained results.

Precision is defined as closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions [3]. It is associated with random errors and is a measure of dispersion or scattering around the mean value, usually expressed by a standard deviation. Depending on the conditions under which the received a series of measurements is obtained, measurement precision can be used to define the following:

- *Repeatability*: measurement precision under a set of repeatable conditions of measurement [3]; precision of results obtained under the same measurement conditions (a given laboratory, analyst, measuring instrument, reagents, etc.).
- *Intermediate precision*: precision of results obtained in a given laboratory over a long-term process of measuring. Intermediate precision is a more general notion (due to the possibility of changes in the greater number of determination parameters) than repeatability [3].
- *Reproducibility*: precision of results obtained by different analysts in different laboratories using a given measurement method [3].

2.3 Uncertainty

Uncertainty is a fundamental property of each measurement. It always occurs at each stage of the analytical procedure. It is necessary to distinguish between:

- *Uncertainty of measurement*: non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used [3]
- *Definitional uncertainty*: component of measurement uncertainty resulting from the finite amount of detail in the definition of a measurand [3]

The basic sources of uncertainty in the course of examination of samples using appropriate analytical procedures are listed in Table 2.1 [4].

Determining the uncertainty of a measurement increases its reliability, and in turn allows comparison of results obtained in interlaboratory studies and helps in deciding the significance of any difference between the obtained result and the reference value. The uncertainty of measurement is a component of uncertainty in all single steps of analytical procedures [5–9]. Therefore, the source of values and uncertainties for individual stages of individual analytical procedure should be specified [10–12].

There are various possible approaches for uncertainty estimation [10–13]:

- *Bottom-up*: based on the identification, quantification, and combination of all individual sources of measurement uncertainty

Table 2.1 Possible sources of uncertainty in the conduct of analysis [4]

Sources of uncertainty	
Personal factors	Instrument factors
Inaccurate or imprecise definition of the measurand	Insufficient resolution of the applied measurement instrument
Lack of representativeness at the step of collecting a sample from an examined material object	Uncertainties associated with the applied standards and/or reference materials
Inappropriate methodology of determinations	Uncertainties of parameters determined in separate measurements and used in calculating the final result, such as physicochemical constants
Personal deviations in reading the analog signals	
Not recognizing the influence of all the external factors on the result of an analytical measurement	Approximations and assumptions associated with using a given instrument, applied during measurement
Uncertainty associated with the calibration of an applied measurement instrument	Fluctuations of the measurement instrument gauge over the course of repeated measurements, with seemingly identical external conditions

- *Fitness-for-purpose*: based on the definition of a single parameter called the fitness function, which takes the form of an algebraic expression and describes the relation between uncertainty and analyte content
- *Top-down*: based on data obtained from interlaboratory studies (precision)
- *Validation-based*: based on interlaboratory or within-laboratory validation processes (precision, trueness, calibration, limit of detection, robustness)
- *Robustness-based*: based on robustness tests from interlaboratory tests

The final result of the analysis consists, therefore, of [13]:

- Determination of the measured value and its unit
  - The result with the expanded uncertainty value ($y \pm U$, along with units for y and U) (where y is result and U is expanded uncertainty)
  - Value of the factor k, for which the expanded uncertainty has been calculated (where k is the coverage factor)

An estimate of uncertainty is one of the necessary parameters of analytical measurement result. Uncertainty is a fundamental property of each measurement. It always occurs at each stage of each measurement procedure. This is not, therefore, a property that gives rise to additional difficulties during the measuring process.

There is a difference between measurement error and uncertainty. Measurement error is the difference between the determined and expected values, and uncertainty is a range into which the expected value may fall within a certain probability. Therefore, the uncertainty cannot be used to correct a measurement result.

2.4 Summary

The main problem during quality assurance and quality control of analytical results arises from insufficient information about the tools used during this process, and about how they are used. First and foremost should be described the statistical tools used, which lie at the heart of metrology.

Results of analytical measurements are a kind of a product of the chemical analyst's work. Both manufactured products (object of analysis) and analytical results must be of an appropriate quality. In addition, the quality of analytical measurements appears to have its own accumulative requirement: the quality of every product is a result of comparison of the obtained value (analytical result) with the reference value (expected, standard, norm, required). In order for the obtained result to be comparable (authoritative, reliable) to the reference value, its (high) quality must be documented and maintained. The quality of analytical results must be assured in the first place before drawing conclusions about the quality of the examined products.

It should be noted that the basic and necessary parameters that characterize an analytical result are traceability and measurement uncertainty. An analytical result without documented traceability and estimated uncertainty is a source of misinformation. These two parameters are the basic requirements of reliable analytical results.

The values of errors and uncertainty strongly depend on the level of analyte content (concentration). High values of these parameters, unacceptable in the case of an analyte content at the percentage level, can be satisfying in the case of trace analysis.

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Chapter 3
Calibration Problems in Trace Analysis

Paweł Kościelniak, Marcin Wieczorek, and Joanna Kozak

3.1 Analytical Calibration

An entire analytical procedure (i.e., a group of activities leading to information on either the kind or quantity of a component in the sample assayed) consists of several separate stages. If the final goal of this procedure is a quantitative result, one of the essential stages is analytical calibration.

Calibration is necessary because the quantity (content, concentration) of a given component in a sample cannot be found directly, but only through the signals produced by the measurement instruments dedicated to analytical examination of this component. Under well-defined chemical and instrumental conditions, a definite relationship (calibration dependence) between the analytical signal and the concentration of a component (analyte) exists. The crucial point of calibration is to define this dependence and exploit it for determination of analyte in a sample.

Accurate description of the calibration dependence in a theoretical or even semi-empirical way is extremely difficult, perhaps impossible, to achieve. Therefore, the conventional and generally accepted approach is empirical, involving experimental reconstruction of the calibration dependence in the form of a calibration graph. For this purpose, the signals for known concentrations of analyte in standard solutions (i.e., for solutions of known concentrations of analyte) are measured. Then, the signal measured for the analyte in the sample is related to the calibration graph and the analyte concentration calculated.

The problem is that calibration dependence is not a general feature of the analyte and measurement system used, but depends on the chemical environment of the analyte in the sample analyzed. Some of the sample components might influence (interfere with) the analytical signal, causing the so-called interference effect. In particular, interferents can react with the analyte and thus change the angle of the

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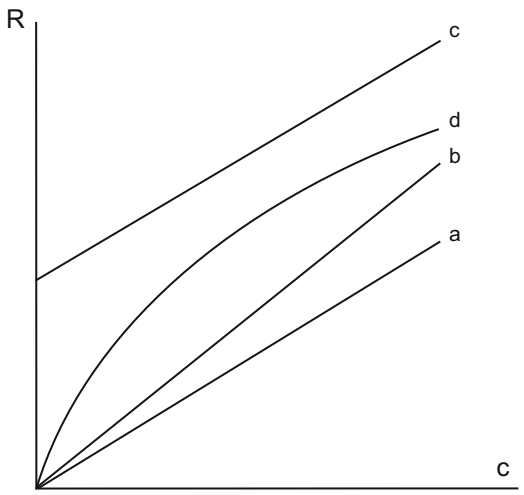

Fig. 3.1 Calibration dependence (*a*) and its change caused by interferents creating multiplicative (*b*), additive (*c*) and complex (*d*) effects

calibration dependence, as presented in Fig. 3.1. In other cases, usually met in spectrophotometric measurements, an effect of nonproportional (multiplicative) but additive character can manifest. In practice, calibration dependence can be even more complex (e.g., nonlinear) because a sample could contain various components that influence the analytical signal in different ways and with different intensities.

If the interference effect occurs in the analytical system used, the empirical reconstruction of calibration dependence through the calibration graph can be a difficult task. Consequently, if this dependence is not reconstructed exactly, the signal measured for the analyte in a sample is in fact related to the "wrong" calibration graph and the analytical result is, more or less, different from the true analyte concentration in the sample (i.e., it is inaccurate).

Risk of occurrence of the interference effect increases with an increasing number of sample components as well as with a higher ratio of the concentrations of interferent and analyte. Because a specific feature of trace analysis is the excess of some sample components accompanying the analyte, the interference effect in these circumstances can potentially lead to great analytical errors.

The analyst must therefore take into consideration the interference effect over the course of the entire analytical procedure and, if necessary, apply "on the way" different procedures to help in its elimination or at least its reduction. The simplest way is to isolate the interferents from the analyte by using such techniques as precipitation and extraction or by exploiting separation (chromatographic, electrophoretic) analytical systems. In other cases, chemical elimination is possible by dosing a sample with special substances that react with interferents. However, if the interference effect is still suspected after the preparative stages, all that can be done

is to try to reconstruct exactly the calibration dependence by applying an appropriately selected calibration approach.

3.2 Calibration Methods

In analytical chemistry, several calibration methods have been developed and introduced into laboratory practice [1]. Most of them can be recommended for use in trace analysis. The methods differ from each other in terms of (a) preparation of the calibration solutions, (b) interpretation of the measurement results and construction of calibration graphs, and (c) calculation of the final analytical results. We present the calibration methods exploited most often in chemical analysis and point out their advantages and drawbacks, which are especially significant from the point of view of the specific conditions characterizing trace analysis.

3.2.1 Set of Standards Method

The most popular approach to calibration is the set of standards method (also called the calibration curve method). It is exploited so commonly and routinely that it is often not even given a special name, and any other calibration approach is taken into account as possible to be used. The method relies on preparation of a set of standard solutions of increasing analyte concentration in the range encompassing the expected concentration of the analyte in the sample. The calibration graph is constructed on the basis of the analytical signals measured for those solutions, as presented in Fig. 3.2. Then, under the same experimental conditions, the signal for the sample is measured and related to the calibration graph. The analytical result is calculated in an interpolative way.

If the standards contain analyte alone (as is usual), such a calibration procedure allows accurate reconstruction of the calibration dependence only when the interference effect does not exist. However, if the sample contains some interferents, the reconstruction cannot be accurate and, consequently, the analyte is determined with a systematic error (as shown in Fig. 3.3).

In principle, there are two general ways of ensuring that the set of standards method leads to an accurate analytical result when the interference effect occurs. The first approach consists of introducing all interferents to the standards in the same amount as present in the sample. Evidently, this is usually a very difficult task, which is practically possible to perform only in the case of samples containing a small number of well-recognized interferents. The second approach is more practicable and reliable and relies on addition of some special reagents to the sample and/or to the standards. The role of these reagents is either to eliminate chemically the influence of the interferents on the analyte or to bring the interference effect to the same level in the samples and in the standards (an example is the buffering of

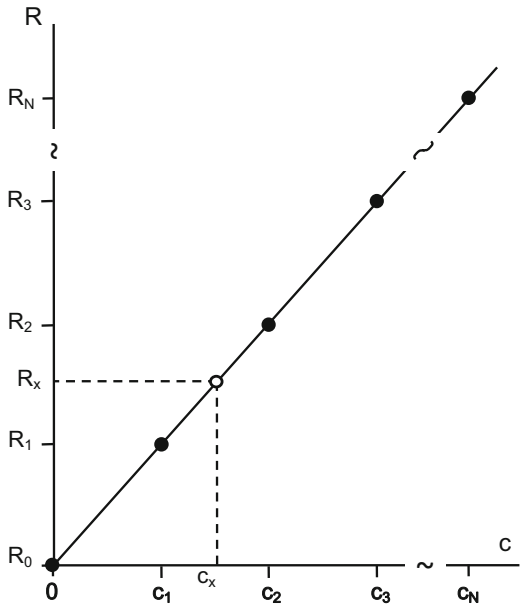

Fig. 3.2 Set of standards method: R_0–R_N signals measured for a set of standard solutions, c_1–c_N analyte concentrations in the standard solutions, R_x signal measured for a sample, c_x calculated analyte concentration in the sample

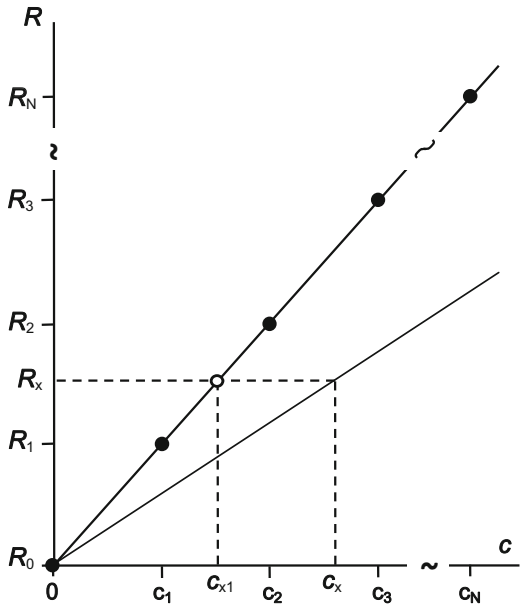

Fig. 3.3 Systematic error in the set of standards method: c_x expected analyte concentration, c_{x1} concentration found as the result of incorrect reconstruction of the calibration dependence

spectral conditions by addition of an excess of alkali metals in flame atomic spectrometry). However, such a procedure is limited by the number and kind of reagents that can act effectively in various analyte–interferent systems.

The evident advantages of the set of standards method is its simplicity and great efficiency, as the calibration graph, once constructed, can serve for analyte determination in many samples. This is the main reason for its popularity among analysts. However, as described above, the method has to be applied with special caution, especially in trace analysis. Therefore, it must be carried out in strict accordance with developed and well-verified analytical procedures. Otherwise, it can be used only after diagnostic tests confirm the absence of interferents in the sample analyzed or indicate how the interference effect can be eliminated.

3.2.2 Standard Addition Method

In contrast to the set of standards method, in the standard addition method a set of standard solutions of increased analyte concentrations are added to sample portions of equal volumes, leaving one of the sample portions free of added standard. After dilution of all calibration solutions to the same volume, the signals are measured. The signals correspond to the sum of the analyte concentration in the sample and in the added standard. As the total analyte concentration in every solution is still unknown, the measurement data are presented as a function of increasing concentrations of analyte in the standards (Fig. 3.4). As a consequence, the calibration graph is constructed over a limited range and the analytical result is calculated by extrapolation of this graph to the zero signal value.

The most important advantage of this calibration approach is that each calibration solution contains the analyte in the environment of all sample components, including potential interferents. Thus, if the calibration dependence is distorted as a result of interferents, there is still a chance to reconstruct it accurately by means of the calibration graph. From this point of view, the standard addition method is one way to eliminate (or rather compensate for) the interference effect. Furthermore, it gives a chance to compensate for the effects of all interferents, independently of their kind, number, and concentration in the sample. This feature supports application of the standard addition method to trace analysis.

Unfortunately, the method suffers several significant drawbacks, which should also be taken into account in trace analysis [2]. First, in principle, the method leads to greater random analytical errors than the set of standards method when both calibration approaches are performed under the same experimental conditions. Second, because the method is based on extrapolation, in some circumstances it can be also a source of serious systematic errors.

These problems are related to the fact that the calibration dependence is reconstructed in the extrapolation part by the same function (usually linear) that fits the measurement points in the experimental range. However, because the extrapolation part corresponds to an excess of the interferents in relation to the analyte, it can in reality be of quite different shape (i.e., nonlinear). Similar

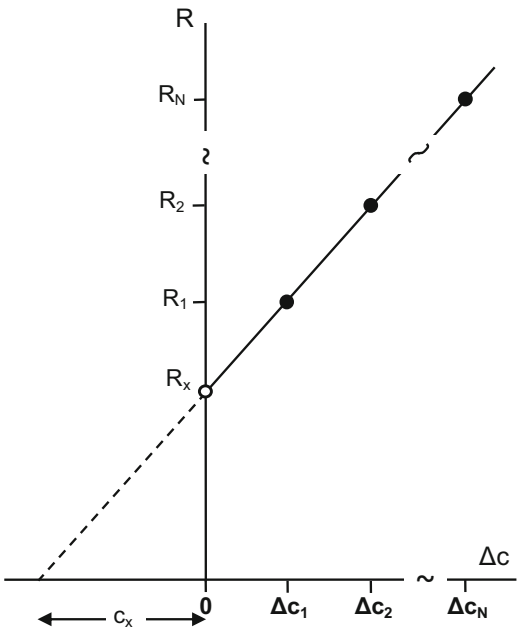

Fig. 3.4 Standard addition method: R_1–R_N signals measured for a sample with a set of standard additions, Δc_1–Δc_N increases in analyte concentration in a sample caused by standard additions, R_x signal measured for the sample, c_x calculated analyte concentration in the sample

difficulty occurs when the chemical form of the native analyte (present in the sample) is different from the form added to the sample. If both forms of analyte are influenced by interferents, the extent of the interference effect can be quite different and, as the result, the calibration dependence can be described by different functions. Figure 3.5 shows that the analyte concentration found by extrapolation in both cases is quite far from the expected result.

Because of the problems described above, the standard addition method can only be recommended in trace analysis with serious reservation. However, the possibility of compensating for the interference effect, even when unexpected or caused by unknown sample components, is so great an advantage that this calibration approach deserves greater interest in analytical practice than it is given at present.

3.2.3 Internal Standard Method

Another calibration approach is the internal standard method. It can be applied when it is possible to measure the analytical signals for two sample components simultaneously. The calibration solutions are prepared in such a way that an internal standard of constant and known concentration is added to both the standard solutions of increasing analyte concentration and to the sample being assayed. All

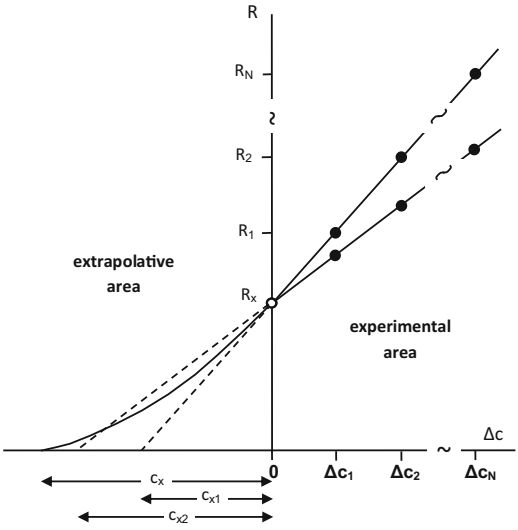

Fig. 3.5 Systematic errors in the standard addition method: c_x analyte concentration expected when the calibration dependence is nonlinear in the extrapolation region, c_{x1} concentration found when the calibration dependence is reconstructed by a linear calibration graph, c_{x2} concentration found when analyte in a sample is of different chemical form to that of analyte added to the sample

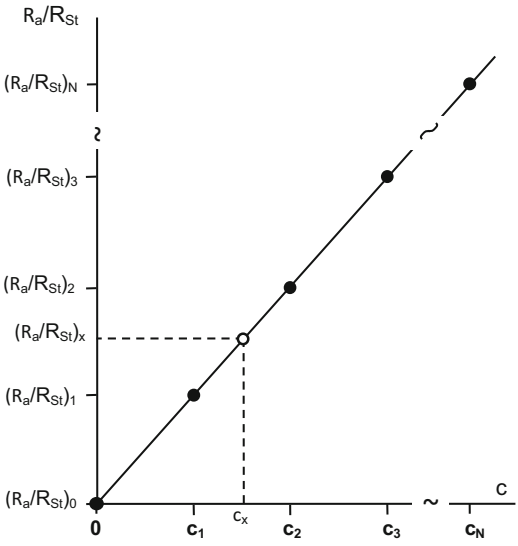

Fig. 3.6 Internal standard method: $(R_a/R_{st})_0$–$(R_a/R_{st})_N$ signal ratios measured for analyte and internal standard in a set of standard solutions, c_1–c_N analyte concentrations in the standard solutions, $(R_a/R_{st})_x$ signal ratio measured for analyte and internal standard in the sample, c_x calculated analyte concentration in the sample

solutions are then measured in conditions characteristic for the analyte and for the internal standard. The calibration graph is constructed as the relationship of the ratio of both signals to the analyte concentration, and the analytical result is calculated in an interpolative way (Fig. 3.6).

The fundamental advantage of the internal standard method is the possibility of increasing the precision of the analytical result, as the signal ratio can be measured with greater precision than individual signals. An essential condition is that the

internal standard is susceptible to signal fluctuations (in terms of their values and direction) to the same extent as the analyte. Therefore, the internal standard has to be chosen very carefully in each case, preferably on the basis of initial experiments or from literature data. Furthermore, it is clear that the internal standard cannot be a substance that is either present in the sample or able to react with sample components.

Unfortunately, the resistance of the internal standard method to the interference effect is, in principle, even less than for the set of standards method. The reason is that the sample components can change both the signal measured for the analyte and the signal measured for the internal standard. The chance that that both changes are the same (or at least similar) is very small, especially in trace analysis. Therefore, the method can be exploited only if the interferences (if present) are eliminated in one of the ways applied for calibration by the set of standards method.

3.2.4 Indirect Method

When the analytical instrument used is not able to directly produce signals for the analyte but only for a substance able to react with the analyte, calibration by the indirect method is used. The reagent is added in a constant and known amount to both sample and standard solutions. If it is added in excess in relation to the highest analyte concentration, the amount remaining after reaction decreases in successive standard solutions. The calibration graph produced is presented in Fig. 3.7.

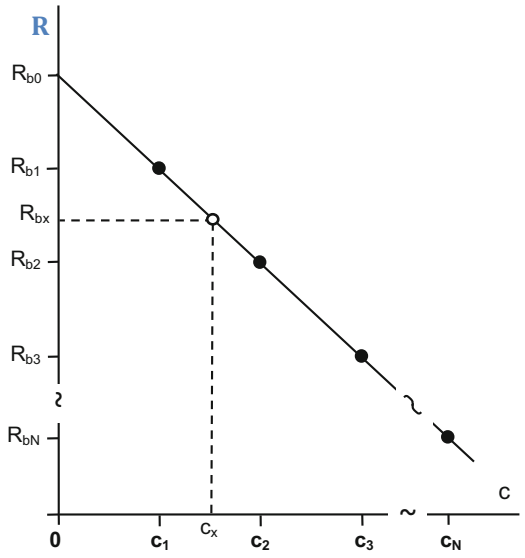

Fig. 3.7 Indirect method: R_{b0}–R_{bN} signals measured for the reagent present in a set of standard solutions, c_1–c_N analyte concentrations in the standard solutions, R_{bx} signal measured for the reagent present in the sample, c_x calculated analyte concentration in the sample

The indirect method is a calibration approach complementary to the set of standards method in the sense that it allows determination of additional analytes with the use of a given detection system. For instance, it is often used for the determination of many anions in trace amounts by atomic absorption spectrometry [3]. It is interesting that the method gives a chance to "positively" exploit the interference effect. Reaction between the interferent and analyte in the set of standards method can be exploited such that the signal is measured for the analyte (now considered the reagent) and the interferent takes the role of analyte.

However, the interference effect is still a very serious problem in the indirect method because sample components can change the analytical signal, not only by direct influence on the reagent, but also indirectly by reaction with the analyte. Because the standard solutions are prepared and measured separately from the sample, systematic error of the analytical result can be expected in both cases unless the appropriate measures (mentioned above) are taken to eliminate interferences.

3.2.5 Dilution Method

The least used calibration approach is the dilution method. This involves gradual dilution of the sample and the standard solution and interpolative calculation of the analyte concentration (i.e., apparent concentration) at each step of the dilution process (Fig. 3.8a). Because the set of apparent concentrations are, in principle, statistically equal to each other, the analytical result is calculated as their mean value.

When the sample contains interferents, their influence on the analyte in the sample is progressively diluted and usually changes. As a consequence, the apparent concentrations are also changed and approaches nonlinearly at a certain concentration value, which is assumed as the final analytical result (Fig. 3.8b). This value is calculated by extrapolation of the nonlinear function fitted to the experimental points. Such a method is not favorable for good analytical precision and accuracy, which is the most serious drawback of the method. On the other hand, if the interference effect is diminished in the course of sample dilution (which is often the case), the analytical result has a chance to be free of this effect and to be accurate without any other additional efforts.

In trace analysis, the problem with the dilution method is that the low initial analyte concentration becomes even lower with progressive dilution of the sample. Therefore, it can be applied only when the analyte is measured with sufficiently high sensitivity, allowing the sample to be diluted a few times at least.

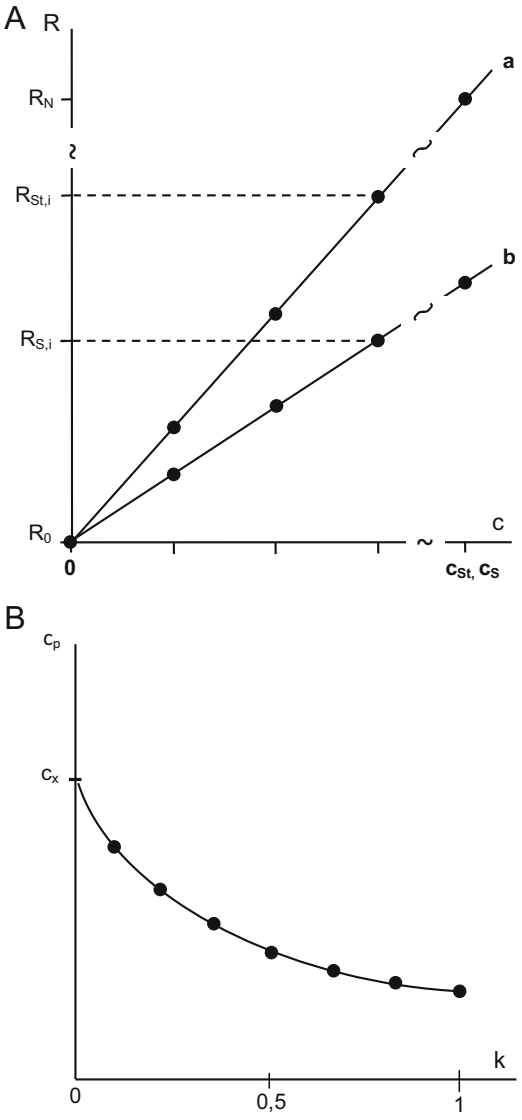

Fig. 3.8 Dilution method: (**a**) Calibration curves for standard (*a*) and sample (*b*) solutions; c_{St} and c_S initial analyte concentrations in the standard and sample solutions, respectively; $R_{St,i}$ and $R_{S,i}$ signals measured for the standard and sample solutions, both diluted to the same extent. (**b**) Relationship between apparent concentration c_p and dilution factor k; c_x calculated analyte concentration in the sample

3.3 Calibration in Flow Analysis

The proper implementation of calibration is to a large extent determined by careful and correct preparation of calibration solutions and samples for measurements. This is especially important in trace analysis because even the smallest errors, random or systematic, at the laboratory stage of the calibration procedure can significantly influence the precision and accuracy of the obtained results.

If this condition is not complied with, the cause is not generally lack of adequate awareness and knowledge, but mostly reasons of a practical nature. Preparing a series of calibration solutions and introducing relevant components (e.g., eliminating the interference effect) or adding standards to the examined sample (with simultaneous attention to the appropriate purity of all reagents and solutions) are labor- and often cost-consuming activities, which always considerably extend the time of analysis. This problem becomes even more important with a greater number of samples is to be analyzed or when the qualitative composition of the samples is more complex.

The techniques used in flow analysis can help overcome these problems. The natural peculiarities of flow techniques are conducive to proper and efficient preparation of calibration solutions and, therefore, in obtaining reliable analytical results in trace analysis. These techniques have been successfully examined and are widely employed for a range of analytical purposes. They are also widely used to perform calibration by a variety of procedures, implementation of which in the traditional way is not possible.

Characteristics of Flow Analysis In flow analysis, solutions are brought to the measuring instrument in flow mode through narrow (internal diameter of a fraction of a millimeter) Teflon tubes toward the detector (Fig. 3.9). If several solutions (e.g., sample and reagents) are needed, they are introduced into separate tubes that are joined together before the detection module. Flow of the solutions is enforced by relevant pumps (usually peristaltic or syringe), which enable regulation and control of the flow rate of each solution separately. During the flow, solutions can be merged with each other or exposed to more complex treatments (digestion, extraction, etc.). In such a flow system, valves (mechanical or electromagnetic), which serve to change the flow of the solution from one tube to another, can also be installed. These valves can also be used to collect a defined volume from the stream of a selected solution (segment, usually of volume of several dozen microliters) and to introduce (inject) it to another solution (flow injection analysis technique). The flow system can be extended with additional tubes and other modules, depending on the concept adopted for its use in a certain analytical application.

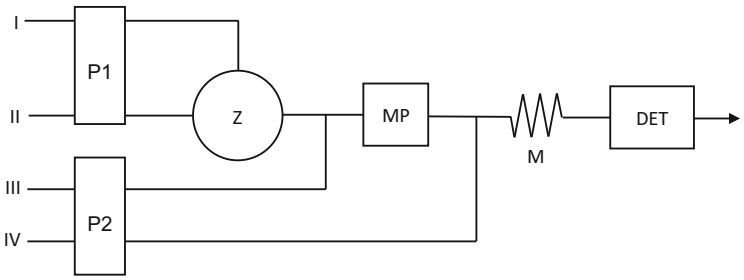

Fig. 3.9 Scheme of typical flow system: *I*, *II*, *III*, *IV* solutions (e.g., sample, standard, reagents), *P1* and *P2* pumps, *Z* valve, *MP* sample preparation module, *M* mixing coil, *DET* detection system

From the point of view of trace analysis, the fact of bringing solutions to a measuring instrument in closed tubes is very important, because this limits the risk of contamination of these solutions during the entire analytical procedure. By using narrow-bore tubes, as well as dosing valves, the volume of solutions used can be much smaller than in the case of analyses performed in the traditional way. The possibility of performing various treatments to prepare a sample for measurement during its flow to the detection system greatly improves and accelerates the analysis. In addition, the strictly determined sequence and synchronization of all processes occurring in the flow system promotes very good repeatability and reproducibility of these processes, which in turn, can improve the precision of the analytical results.

The flow system shown in Fig. 3.9 can be adapted to calibration tasks. It is easy to imagine that the sample to be analyzed is introduced into one of the tubes, injected into another solution (e.g., water), undergoes digestion (e.g., by means of ultraviolet radiation), is merged and mixed with selected reagents that eliminate the interference effect, and in the end is measured in the detection system. Similarly, the instrument can measure all pre-prepared calibration solutions (although subjecting them to digestion and combination with reagents is redundant, the equal treatment of all solutions is conductive to the achievement of precise and accurate analytical results).

In this way, it is possible to collect measurement data enabling calibration using the set of standards method. In order to implement other calibration methods, the tube installed directly behind the valve can be used to deliver standard solutions to the injected segment of sample (standard addition method), or to merge with the injected solutions of sample and standards the internal standard solution (the internal standard method) or an appropriate reagent (indirect method). In these cases, the flow technique contributes to more efficient and economic preparation of complex calibration solutions than the traditional mode.

Selected Calibration Procedures in Flow Analysis In recent decades there have been great developments in various fields of flow analysis, including a number of interesting and specific proposals for the flow mode of analysis calibration . These allow implementation of the various methods of calibration in accordance with procedures that are often completely different from those that analysts use in their everyday work [4, 5]. The advantage of these approaches compared with the procedures "existing" in traditional analysis relies not only on more efficient, automated implementation of full calibration (and thus also the complete analytical procedure), but also on the opportunities for more efficient use of the registered analytical signals to obtain richer measurement information.

One of the tasks that can be accomplished relatively easily in flow analysis is the creation of conditions that allow a series of measurement results to be obtained using only a single standard solution (and not several standard solutions). A classic example is the network calibration method, performed using a flow system made up of a network of tubes of different lengths connected to each other prior to the detector (Fig. 3.10a) [6]. In this system, the injected segment of standard solution is

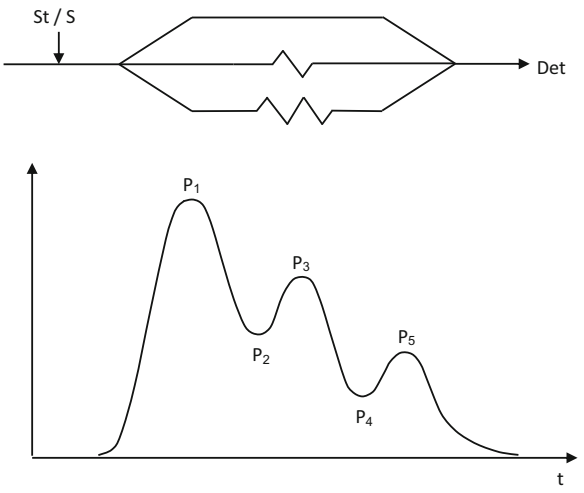

Fig. 3.10 Network calibration method: (**a**) *St* standard solution, *S* sample solution, *Det* detection system; (**b**) P_1–P_5 characteristic points serving for construction of the calibration graphs (see Fig. 3.11); *t* time after injection

divided into several parts (e.g., three), each of which reaches the detector at a different time because they follow paths of varying length. If the flow rate and length of the tubes are chosen so that particular parts of the original segment partially overlap each other, the measurement image consists of three overlapping peaks such that five characteristic points can be distinguished (Fig. 3.10b). After injection of sample to a system of the same parameters, a similarly complex signal is obtained, which is proportionally lower than the signal registered for the standard at all five points.

The calibration method described above is a version of the set of standards method, in which analytical signals are not differentiated by the use of calibration solutions containing different concentrations of the analyte, but as a result of changes in flow conditions of a single standard solution. Therefore, interpretation of the measurement results is also different, as shown in Fig. 3.11. Values of signals obtained for different segments of the standard solution are attributed to the concentration of the analyte in the standard, and "two-point" straight calibration graphs can be constructed. Appropriate signals obtained for the sample segments are then referred to the graphs and four estimates of the analyte concentration in the sample are determined, whose mean is a measure of the final analytical result.

In recent years, many flow systems consisting of several pumps (multipump flow systems) or several injectors (multicommuted flow systems) have been constructed and exploited. Some of them are dedicated to calibration purposes. For instance, the standard addition method was recently implemented using a multicommuted system with an injection valve and three directive solenoid valves [7]. The system can automatically prepare a series of calibration solutions by successively merging a sample with three standards of different analyte concentrations. The approach is especially suitable for trace analysis because, according to the author's recommendation, it can be used when the analyte concentration in the sample is below the lower limit of linear response of a detector (it allows, in particular, potentiometric

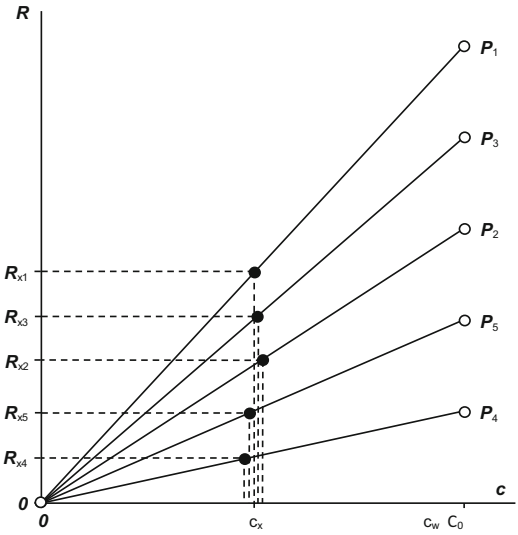

Fig. 3.11 Interpretation of the measurement data obtained in the network calibration method: P_1–P_5 signals obtained for the set of standard solutions, R_{x1}–R_{x5} signals obtained for the sample, c_w analyte concentration in the standard solution, c_x calculated analyte concentration in the sample

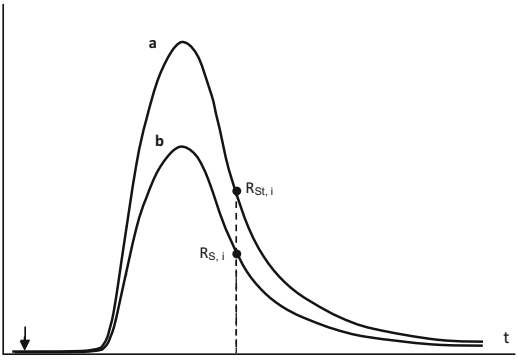

Fig. 3.12 Principle of the gradient ratio calibration method: $R_{St,i}$ and $R_{S,i}$ measurement points registered for the standard solution (*a*) and for the sample (*b*) at the same time point after injection of both solutions (*arrow*)

determination of fluoride at one tenth the concentration of the lower limit of linear response for a fluoride-selective electrode).

Another group of calibration solutions are based on the fact that, in flow-injection analysis, the analytical signal is recorded in the form of a characteristic asymmetric peak (Fig. 3.12), which is the measurement image corresponding to local analyte concentrations in the segment injected into the carrier solution. It is assumed that two solutions of different analyte concentrations, injected into a system one after another (with the same instrumental conditions), give rise to peaks of equal width at their bases (i.e., appearing and disappearing, respectively, at the same time after injection). It can also be proved that both peaks differ from each other in that the two measurement points of the first and second peaks, respectively, registered at the same time after injection of both solutions, are proportional to the concentration of analyte located in each segment at this time point in the same place.

An example of use of the above rules for calibration purposes is a procedure called the method of gradient ratios (gradient ratio calibration method, GRCM) [8], which is one way of implementing the dilution method in flow analysis. In this approach, sample and standard solutions are injected one after the other into a flow system. The obtained peaks overlap each other (as shown in Fig. 3.12) and are considered point-by-point from the maximum points along the softer sides of both peaks. On the basis of the values of the two measurement points obtained for standard and sample after the same time, analyte concentrations can be determined in various parts of the sample segment. For the final analytical result, the average of these concentrations is taken (only if their values differ from each other randomly).

In the GRCM method, it is assumed that analyte concentrations in various parts of the sample segment can be different from each other and from the real concentration, as a result of the interference effect. However, if it is assumed that the considered side of the peak registered for the sample is in fact an image of increasingly diluted sample, and that interferences decrease during sample dilution, then the subsequent calculated values of local concentration approach the true analyte concentration in the sample. In practice, this concentration is determined (as shown in Fig. 3.8b) by expressing the values of local concentration as a function of sample dilution and extrapolating this function to the value corresponding to infinitely great sample dilution.

3.4 Good Calibration Practice in Trace Analysis

In the light of what has been said above, calibration is a process that largely conditions the reliability of analytical results in trace analysis. It is therefore important to bear in mind individual calibration steps, especially those concerning the measurement step, when deciding to perform an analysis according to a specific procedure. One should remember, inter alia, that all calibration solutions should be measured under the same experimental conditions (both instrumental and chemical). The preparation of these solutions for measurement should therefore be performed ably and quickly. Moreover, as far as possible, all preparation procedures should be applied to both calibration solutions and samples so that any uncontrolled loss or gain of analyte occurs in all solutions equally. If maintaining these conditions is difficult or too time consuming, it is advisable to adapt a selected analytical procedure for realization in a flow mode.

As a rule, it is unacceptable to perform analyses on the basis of a calibration graph constructed on a different day. Furthermore, in the case of continued analyses, calibration should be periodically repeated during the day. One should bear in mind that an analytical signal can change considerably under the influence of even slight changes in instrumental parameters. Because this phenomenon can occur even with the use of analytical instruments of very high quality, one should take care not to be misled by a sense of reliability (always apparent to some extent) resulting from their appearance, price, or manufacturer's reputation.

In order to obtain results of the highest precision and the lowest limit of quantification, the experimental conditions should be chosen such that the highest signal possible is measured for an analyte in a sample. It is also important that the established analytical procedure is the least complex, as far as possible, because every single physical or chemical operation a sample has to undergo is a potential source of random measurement errors. In the case of trace analysis, situations involving possible substantial loss of analyte from a sample (e.g., as a result of mismatched extraction conditions) or considerable sample dilution (e.g., through addition of essential reagents in an extensive volume) should be especially avoided.

Accuracy of the obtained results is determined first of all by whether the measurements made for an analyte are vitiated by an interference effect. It is therefore of vital importance to judge well whether the sample contains components that could act as interferents with respect to the analyte. If there are any doubts in this matter, and they are not dispelled by the analyst's own experience or the proceedings of other researchers, analyses can be performed on the basis of the standard addition method (taking into account the limitations of the method). However, some additional efforts can also be made to examine the situation experimentally.

Examination of the Interference Effect The most usual way of examining the interference effect is very simple; it is based on performing measurements for the analyte alone at a given concentration, and for the analyte at the same concentration in the presence of an interferent. This enables detection, one by one, of the interactions of the analyte with all the substances composing the sample. Certainly, this approach is tedious, time consuming, and does not ensure that all components are taken into consideration. One should also remember that the chemical form of an individual component in a standard solution might be different from that present in a natural sample, which can result in them having different interactions with the analyte.

The above-mentioned method of interference detection has another drawback; it does not allow conclusions to be made about the interaction of a given component with the analyte when the component is in the presence of other interferents. If the number of potential interferents is not very high, it is advisable to examine changes in the signal triggered by the analyte in synthetic solutions containing all components at different concentration levels. Composition of the solutions should be chosen on the basis of careful experimental design to give a wealth of information on the simple and complex interactions of interferents with an analyte using a relatively small number of solutions [9].

One simple way of identifying the interference effect is often forgotten in analytical practice. It is based on performing measurements for three solutions: sample alone, analyte at a certain concentration, and sample containing added analyte at the same concentration. On the basis of these three results, two calibration graphs can be prepared, as presented in Fig. 3.13. If a sample contains components producing an interference effect, the graphs have differing slopes; otherwise the graphs are parallel to each other. In this way, one can assess the

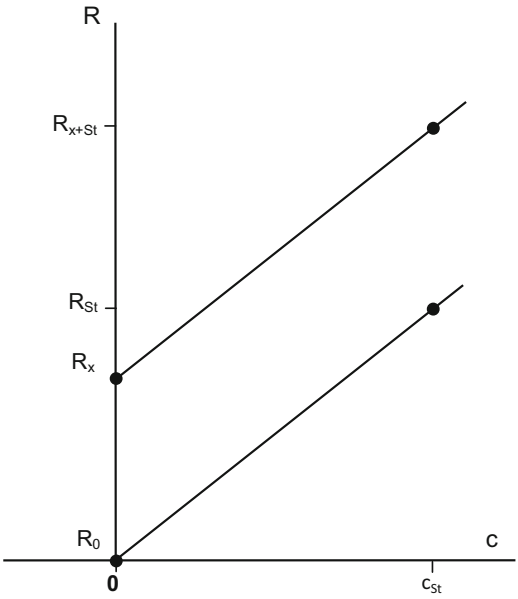

Fig. 3.13 Interference effect examined by comparison of the slopes of two calibration graphs based on the signals obtained for a standard solution (R_{St}) containing a known analyte concentration (c_{St}), the sample (R_x), and the sample with added standard (R_{x+St})

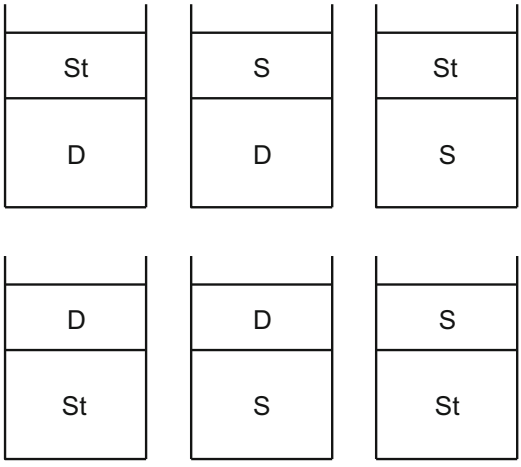

Fig. 3.14 Set of solutions prepared according to the complementary dilution method, containing diluent (*D*), standard (*St*), and sample (*S*)

resultant interaction of all interferents (producing the multiplicative effect) present in a sample, regardless of their number, kind, and concentration. Simultaneously, the obtained results indicate whether the set of standards method is sufficient or whether the standard addition method should be applied.

The complementary dilution method (CDM) [10] is a development of the above-mentioned method for examination of the interference effect. It requires preliminary preparation of six solutions according to the scheme given in Fig. 3.14. Once measurements for the analyte in all the solutions have been completed, four

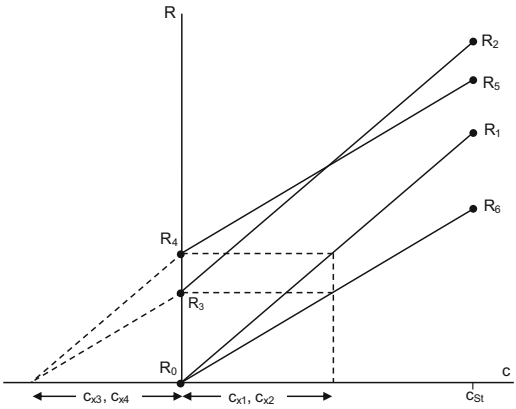

Fig. 3.15 Calibration graphs based on the signals (R_0–R_6), obtained for the solutions presented in Fig. 3.14, allowing analyte concentration in the sample to be calculated by interpolation (c_{x1}, c_{x2}) and extrapolation (c_{x3}, c_{x4})

calibration graphs can be prepared, as illustrated in Fig. 3.15. Mutual inclination of the graphs is different in pairs because the analyte is present in the respective solutions at different concentration levels. Interpretation of the graphs is made not on the basis of their mutual inclination, but via comparison of the four analytical results obtained from the graphs.

It should be noted here that two of the four results are obtained by interpolation (as in the set of standards method) and two by extrapolation (according to the standard addition method). All the results are estimated values of the analyte concentration in the sample. Therefore, if the obtained results differ from each other there is a high probability that an interference effect occurs. Nevertheless, results obtained by extrapolation are closer to the real analyte content in the sample than those calculated interpolatively.

In order to improve the procedure described above it was adapted to flow mode with the use of an original flow-injection manifold [11]. Once a sample and a single standard solution are introduced to the system they can be gradually diluted, and interpolative and extrapolative estimations of analyte concentration in the sample can be obtained for every dilution degree. This procedure allows examination of possible interferences, and is, in effect, a sterling, integrated calibration method that combines in one calibration procedure the set of standards method and the standard addition method with the dilution method. Moreover, it has been demonstrated that any of the calibration methods described above can be integrated with the use of a constructed manifold.

Specific Calibration Conditions In analytical practice there are certain situations in which reliable analytical results can be obtained for an analyzed sample even in the presence of components producing an interference effect. Success depends on the way calibration is performed, especially on preparation of the calibration solutions or application of a suitable calibration approach.

In the vast majority of cases, calibration is performed in such a way that standard solutions are prepared independently of sample solutions. The guiding principle is an attempt to copy, as far as possible, the composition of samples in standard

solutions. On the other hand, introduction of additional components to standard solutions always poses the threat of uncontrollable addition of a certain amount of analyte. However, exact matching of the chemical composition of standards and samples is generally not necessary when one of the components of the sample is present in a large excess relative to the analyte (e.g., in the case of analyses aimed at quality control of chemicals). It is then sufficient to include in the standards only the main component.

Reference materials, that is, materials of known composition of components (usually determined on the basis of interlaboratory tests), can be of great help in proper preparation of standard solutions. They usually serve for verification of trueness of a given analytical procedure and accuracy of the obtained analytical result. If the composition of a reference material matches that of the analyzed sample, and the substance to be analyzed is one of its components, then the material can be utilized for calibration in such a way that a series of standard solutions is prepared via addition of analyte in known quantities.

There is one more, very important and relatively simple method to use when an interference effect is difficult to explore but its occurrence is probable and poses a threat to the reliability of analytical results. This refers to the case when an analyzed series of samples have similar chemical composition (at least in terms of the composition of interferents) and the determined component is present in all samples in a similar quantity. In this situation, the standard addition method can be used for analysis of one selected sample and the constructed calibration graph employed for interpolative determination of analyte in the remaining samples. This combined procedure is depicted in Fig. 3.16. Thus obtained results are, as a rule, more accurate than those obtained after application of the set of standards method to all the samples. In addition, the analyses are conducted faster than when all the samples are analyzed using the standard addition method.

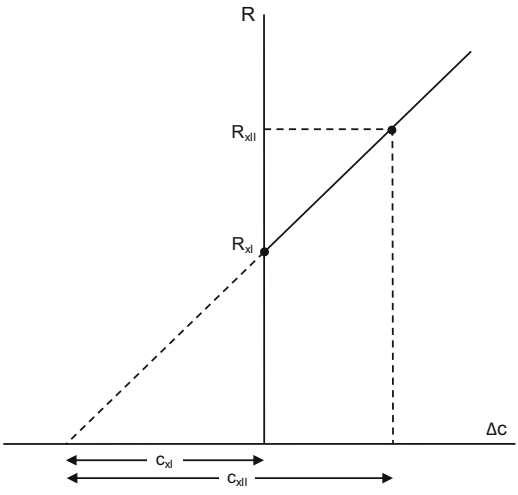

Fig. 3.16 Combined calibration approach: R_{xI} and R_{xII} signals obtained for two samples (*I* and *II*) of similar composition, c_{xI} and c_{xII} analyte concentrations found in samples *I* and *II* by extrapolation and interpolation, respectively

There is also a case when an occurring interference effect is constant, independent of the ratio of interferent concentration to analyte concentration in a sample. This is so, for instance, when interferents are present in a sample in such high concentrations that even considerable reduction of their amount in relation to the amount of analyte does not change the mutual interactions of these components. In this case, the standard addition method can be applied in combination with gradual dilution of calibration solutions. The simplest way is to proceed in accordance with the "sequential standard addition calibration" (S-SAC), which is based on successive addition of a fixed volume of a standard solution to a sample [12]. In another approach, termed the "standard addition and indicative dilution method" (SAIDM), a sample is spiked with a single standard and the solution successively diluted with a neutral diluent until the measured signal equals the signal produced by the undiluted sample [13].

In the same situation, calibration can be performed using the flow injection technique exploiting the "interpolative standard addition method" (ISAM) [14]. In this approach, a series of standard solutions are injected one after another into a stream of sample flowing continuously toward the detector. A steady-state signal is produced by the sample, whereas the standard solutions (diluting the sample to the same degree) cause changes in this signal in the form of peaks (Fig. 3.17a). Standards with concentration of analyte higher than that of the sample produce "positive" peaks and standards with a lower concentration produce "negative" peaks. The difference between the maximal (or minimal) point of these peaks and the steady-state signal is determined, followed by construction of a calibration graph presenting the dependence of the signal difference on the analyte concentration in the standards (Fig. 3.17b). Interpretation of the graph is performed on the basis of the observation that the signal measured for a standard should not cause changes in the steady-state signal only when the analyte concentration in the standard and the sample are equal (i.e., when the signal difference is zero, the

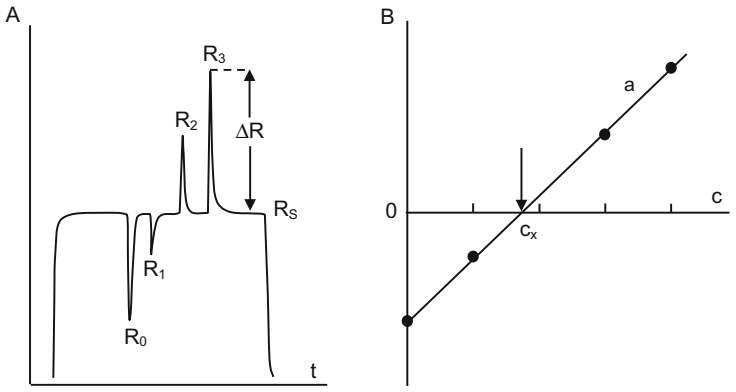

Fig. 3.17 Principle of the interpolative standard addition method: (**a**) R_S signal measured for the sample, R_0–R_3 signals measured for the standard solutions. (**b**) Calibration graph (*a*) obtained by plotting ΔR against sample concentration (*c*); c_x calculated analyte concentration in the sample

concentration of analyte in the standard equals that of the sample). The analytical result is then determined from the point of intersection of the calibration graph and the concentration axis.

3.5 Conclusions

As shown, various calibration methods can be applied in chemical analysis. The choice of method depends on the kind of analytical problems and sources of random errors expected in the course of analysis. Nevertheless, it is hard to say that any of the discussed methods is especially adapted to trace analysis. However, because of its specificity, trace analysis does require special attention in the choice of calibration method, as well as special care in realization of the selected method at every step of the calibration procedure.

The described calibration approaches are those most often used, but do not exhaust the full range of possibilities that can be exploited in chemical analysis, including trace analysis. However, other approaches are very rarely used or are only at the stage of implementation to analytical practice; therefore, it is hard to treat them on an equal footing with more established methods and to characterize them in relation to the requirements of trace analysis. Nonetheless, in the light of the major role of analytical calibration in chemical analysis there is one thing that leaves no doubt: development, implementation, and dissemination of methods and calibration procedures are among the most important and urgent tasks for analysts to tackle.

Development and popularization of calibration methods are largely contingent on the degree of instrumental and methodological advancement of analytical laboratories in the field of flow analysis. As indicated, flow techniques are helpful in efficient and economical preparation of calibration solutions, and also create the conditions for atypical yet useful interpretation of measurement results. Many of the new calibration methods appearing in flow analysis prove that this approach still plays a major inspirational role and is releasing analysts from a routine way of thinking and acting. It is to be hoped that, over time, flow techniques will find increasing recognition and application in our laboratories.

In summary, one can say that from a calibration point of view, flow techniques are recommended as they give greater freedom and possibilities for manipulation at the stage of preparation of calibration solutions. Separation techniques provide assistance in the field of elimination of interference effects. Combination of these instrumental setups with a detection system providing high determination sensitivity and an opportunity for simultaneous detection of signals for different sample components (e.g., with a mass spectrometer or an ICP spectrometer) is therefore the optimum analytical system from the point of view of trace analysis and the demands that this kind of analysis places on the field of calibration.

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Chapter 4
Certified Reference Materials in Inorganic Trace Analysis

Rajmund S. Dybczyński and Halina Polkowska-Motrenko

4.1 Introduction and Some Basic Concepts

One of the characteristic phenomena in the chemical sciences of the second half of the twentieth and the beginning of twenty-first century was rapid development in the analytical chemistry of traces. New purity standards for materials emerged at the beginning of the atomic era. Uranium used as a fuel in nuclear reactors and other materials useed in nuclear technology had to be as fully free as possible of certain elements (e.g., Dy, Gd) that have a high cross-section for the absorption of thermal neutrons. The requirement that the concentration of these and other elements (e.g., in nuclear-grade uranium) should not exceed a few milligrams per kilogram caused fast progress in such techniques as emission spectrography, spectrophotometry, and neutron activation analysis (NAA), as well as in separation and preconcentration methods for elements. Progress in electronics and the advent of semiconductor technology increased purity requirements in the materials sciences. At the same time, progress in the biomedical sciences drew attention to the role of trace elements essential for life (Co, Cu, Fe, I, Mn, Mo, Ni, Se, Sn) or that are toxic (As, Cd, Hg, Pb). The determination of these elements in tissues, body fluids, and so on is often the basis for medical diagnosis and therapy. On the other hand, ecological movements have focused on global environmental pollution. As a result, monitoring of several chemical compounds and trace elements in water, air, soil, and food has become the daily work of several laboratories. In our times, more and more frequently it seems that determination of the total concentration of a given element in the investigated object is not sufficient. The determination of various physical and chemical forms of an element (i.e., speciation analysis) is often required. In this period of globalization, rapid growth of international trade, and introduction of

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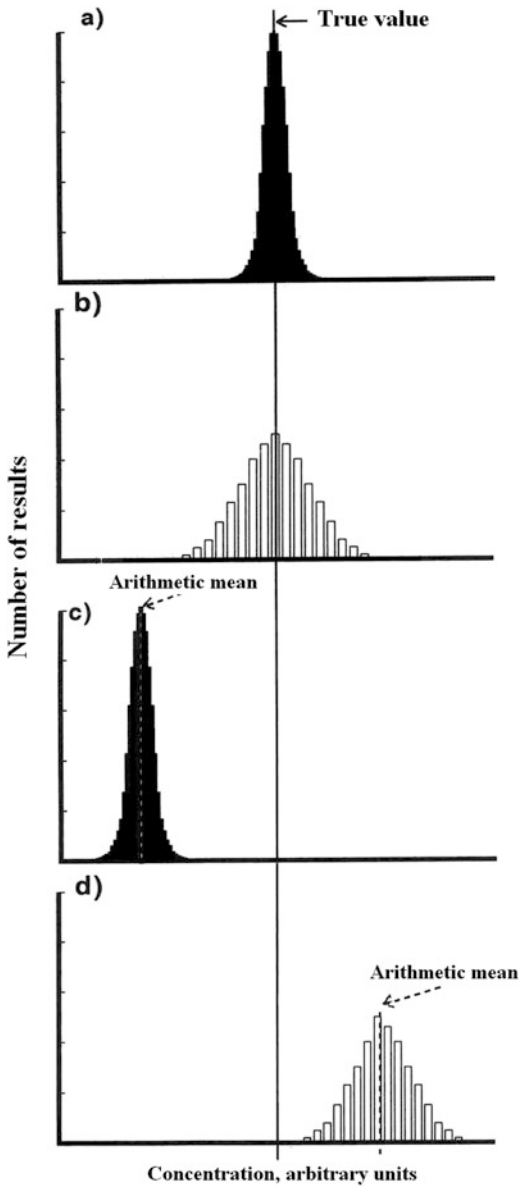

Fig. 4.1 Illustration of the notions of accuracy and precision. (**a**) Method is accurate and precise. (**b**) Method is accurate but of poor precision. (**c**) Method is precise but inaccurate. (**d**) Method is inaccurate and imprecise

international standards, many important decisions (administrative, medical, commercial, technological, and those concerning environment protection) depend on the results of chemical analyses.

Correct decisions can be taken if the analytical results on which they are based are reliable (i.e., they are both accurate and precise). Let us recall a few basic definitions [1]:

Measurement accuracy: closeness of agreement between a measured quantity value and a true quantity value of a measurand

Measurement precision: closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

The notions of accuracy and precision are graphically presented in Fig. 4.1 [2].

4.2 Characteristics of Trace Analysis

It is commonly known that the possibility of making grave errors is much greater in trace analysis than when macroconstituents are being determined. Some spectacular examples of ranges of analytical results sent by laboratories participating in world-wide interlaboratory comparisons (ILCs) are shown in Table 4.1. One should note that differences in results by orders of magnitude occur not only in open comparisons (in which every willing laboratory could participate), but also in cases where the laboratories were hand-picked on the basis of their good reputation and experience.

When macroconstituents are determined, especially in typical kinds of samples, the analyst usually has some idea of what the approximate concentration of a given component should be. A result drastically different from the expectation should be a warning signal that can result in critical evaluation of the procedure and, for example, repetition of the determination. In trace analysis, in general, it is difficult to foresee what the concentration of a trace constituent should be in a given sample and so results differing by orders of magnitude could seem equally probable. It should be remembered that the great potential possibilities offered by modern instrumental analytical techniques, together with assertive advertising of new instruments by the producers, could weaken the sense of criticism of less-experienced analysts. Some tend to consider that a highly automated and computerized instrument costing tens or hundreds of thousands of dollars is a "black box" that must give correct results. It should be remembered that the high price of an apparatus does not automatically eliminate the possibility of making gross errors as a result of matrix effects and various types of interference. Some analysts forget that the process of analysis starts at the stage of sampling, which is followed by sample preparation and sometimes separation/preconcentration steps, and only at the end is the act of measurement. Grave errors can be made in the first stages of an analytical process. The element being determined (analyte) can be lost during sample preparation or during a separation/preconcentration step as a result of volatility, adsorption, etc. It can also be added to the sample because of air pollution or from laboratory vessels, reagents, acids used to dissolve the sample, and so on. It is interesting to note that the large differences in results sent by individual laboratories were observed not only for trace elements usually considered "difficult" to determine (As, Cd, Co, Pb, Se), but also for common elements that occur in considerably

Table 4.1 Selected examples of dispersion of analytical results observed in international interlaboratory comparisons over the last 40 years

Material	Element	Number of laboratories	Range of results	References
Fuel oil	Ag	4[a]	0.006–0.1 mg/kg	von Lehmden et.al. 1974 [3]
	Hg	4[a]	0.005–0.4 mg/kg	
Coal	K	8[a]	20–22,000 mg/kg	von Lehmden et.al. 1974 [3]
Milk powder IAEA A-11	Cd	8	1.10–1660 ng/g	Dybczyński et al. 1980 [4]
	Co	18	0.004–51.5 mg/kg	
	Cr	16	0.016–1160 mg/kg	
Milk powder IAEA A-11	Co	7[a]	3.7–40 ng/g	Byrne et al. 1987 [5]
	Ni	4[a]	22.1–500 ng/g	
	Pb	4[a]	43–300 ng/g	
Whey powder IAEA-155	As	7	1.33–3893 ng/g	Zeiller et al. 1990 [6]
	Cd	25	0.73–38,000 ng/g	
	Co	22	3.41–4980 ng/g	
	Cs	11	0.051–20.1 mg/kg	
	Hg	12	0.75–54,333 ng/g	
	Pb	21	0.025–6.53 mg/kg	
Oriental tobacco leaves CTA-OTL-1	Cr	43	0.038–11.6 mg/kg	Dybczyński et al. 1993, 1996 [7, 8]
	Cs	17	0.117–315 mg/kg	
	Na	43	48.2–8083 mg/kg	
	Ni	36	0.005–22.7 mg/kg	
	Pb	40	0.051–19.5 mg/kg	
Mixed Polish herbs INCT-MPH-2	Al	33	4.25–1095 mg/kg	Dybczyński et.al. 2002, 2004 [9, 10]
	As	37	0.027–1.74 mg/kg	
	Ca	75	465–15,243 mg/kg	
	Fe	93	78.7–825 mg/kg	
	Na	53	170–20,700 mg/kg	
	Pb	69	0.077–14.6 mg/kg	
	Se	14	9.6–720 ng/g	

Corn flour INCT-CF-3	As Cr Fe Pb Se	19 27 72 31 7	0.367–220 ng/g 10–5710 ng/g 9.8–1034 mg/kg 11.6–7455 ng/g 1.0–160 ng/g	Polkowska-Motrenko et al. 2006, 2007 [11, 12]
Oriental basma tobacco leaves INCT-OBTL-5	Cr Fe Pb Sc Se	46 63 53 19 9	0.071–19.52 mg/kg 410.15–2006.7 mg/kg 0.127–9.53 mg/kg 1–753 ng/g 0.045–1.44 mg/kg	Samczyński et al. 2011 [13]
Polish Virginia tobacco leaves INCT-PVTL-6	Ni Pb Th U	36 20 11 8	0.294–68.2 mg/kg 0.066–79.9 mg/kg 64.14–12,485 ng/g 9.37–132.5 ng/g	Samczyński et al. 2011 [14]

[a]Laboratories selected on the basis of good reputation and experience

Table 4.2 Sources of errors in inorganic trace analysis [15]

Most common sources of errors in trace analysis	
Inadequate sampling	
Inhomogeneity of the test sample with respect to trace analysis	
Analyte can be lost from the sample by	Analyte can be added to the sample from
Volatilization Adsorption Incomplete dissolution Failing to transform the analyte into the desired chemical form Nonquantitative recovery during preconcentration and/or separation step	Reagents, acids, solvents, etc. Sampling devices, reaction vessels Glassware, tubing, etc. Ion exchange resins Extractants Adsorbents Water Laboratory air
Instrumental errors	
Matrix interferences	
Blank problems	
Calculation errors	
Trivial errors (e.g., writing erroneous mass or concentration units when reporting the results)	

higher concentrations (Ca, Fe, K, Na) (see Table 4.1). Possible sources of grave errors in trace analysis are shown in Table 4.2 [15]. The magnitude of errors strongly depends on the concentration of the analyte(s). Accuracy and precision obtained for a certain type of sample can be quite satisfactory in the case of constituents present at the milligram per kilogram level but much worse for those at the nanogram per gram level [15, 16].

4.3 Methods for Checking Accuracy

As mentioned earlier, the analyst must take care that the results of analysis are reliable (i.e., both accurate and precise). Good precision of determinations obtained using a given method is not enough reason for uncritical satisfaction. There are known cases in which a laboratory provided very precise results that were, however, far from the true value [8, 11, 15]. Although precision can easily be checked internally, for example, by making several repetitions of the determination and calculation of standard deviation, confidence level, and so on, checking accuracy is a much more difficult task. Several approaches are possible:

(a) Analysis of the sample by two or more analytical methods based on different physico-chemical principles
(b) Participation in ILCs
(c) Comparison of results with those obtained by a method of "guaranteed accuracy" (e.g., definitive or primary)

(d) Use of appropriate certified reference materials (CRMs) of matrix type and analyte(s) concentration similar to those in the analyzed sample

Not all laboratories have appropriate methods (i.e., those with correspondingly good limits of determination and uncertainties) for checking the accuracy of their results "at home" (method a). Participation in an ILC is very helpful and instructive (method b), but the elaboration of results could last months or more, so there is no immediate possibility of checking if or why a laboratory's result(s) deviates markedly from those of other laboratories. The use of definitive methods (method c), that is, primary reference measurement procedures according to the International Organization for Standardization (ISO) [1], is perhaps the most reliable means of verifying the accuracy of results, but unfortunately definitive methods are available only in highly specialized laboratories.

The use of CRMs (method d) is accessible for most laboratories. If an analyst determining an analyte (e.g., an element in the CRM) is obtaining correct results (i.e., in agreement with the certificate) it can be expected that the results of determinations of this element in other samples are also reliable. The requirement, however, is that the general composition of the analyzed samples and the concentration of the element(s) being determined are similar to those in the CRM.

4.4 Reference Materials and Certified Reference Materials

Definitions

The most recent definitions of reference materials (RM) and certified reference materials (CRM) were given in the *International vocabulary of metrology – basic and general concepts and associated terms (VIM3)* [1].

Reference Material

Material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties.

Note 1: Examination of a nominal property provides a nominal property value and associated uncertainty. This uncertainty is not a measurement uncertainty.

Note 2: RMs with or without assigned quantity values can be used for measurement precision control, whereas only RMs with assigned quantity values can be used for calibration or measurement trueness control.

Note 3: "Reference material" comprises materials embodying quantities as well as nominal properties.

Example 1: Examples of RMs embodying quantities:

(a) Water of stated purity, the dynamic viscosity of which is used to calibrate viscometers

(b) Human serum without an assigned quantity value for the amount-of-substance concentration of the inherent cholesterol, used only as a measurement precision control material
(c) Fish tissue containing a stated mass fraction of a dioxin, used as a calibrator

Example 2: Examples of RMs embodying nominal properties:

(a) Color chart indicating one or more specified colors
(b) DNA compound containing a specified nucleotide sequence
(c) Urine containing 19-androstenedione

Note 4 An RM is sometimes incorporated into a specially fabricated device.

Example 1: Substance of known triple-point in a triple-point cell
Example 2: Glass of known optical density in a transmission filter holder
Example 3: Spheres of uniform size mounted on a microscope slide.

Note 5: Some RMs have assigned quantity values that are metrologically traceable to a measurement unit outside a system of units. Such materials include vaccines to which International Units (IU) have been assigned by the World Health Organization.
Note 6: In a given measurement, a given RM can only be used for either calibration or quality assurance.
Note 7: The specifications of a RM should include its material traceability, indicating its origin and processing [17].
Note 8: ISO/REMCO has an analogous definition [17] but uses the term "measurement process" to mean "examination" (ISO 15189:2007, 3.4), which covers both measurement of a quantity and examination of a nominal property.

Certified Reference Material
Reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures.

Example: Human serum with assigned quantity value for the concentration of cholesterol and associated measurement uncertainty stated in an accompanying certificate, used as a calibrator or measurement trueness control material.

Note 1: "Documentation" is given in the form of a "certificate" (see ISO Guide 31:2000 [28]).
Note 2: Procedures for the production and certification of CRMs are given (e.g., in ISO Guide 34 [31] and ISO Guide 35 [32]).
Note 3: In this definition, "uncertainty" covers both "measurement uncertainty" and "uncertainty associated with the value of a nominal property," such as for identity and sequence. "Traceability" covers both "metrological traceability of a quantity value" and "traceability of a nominal property value."
Note 4: Specified quantity values of CRMs require metrological traceability with associated measurement uncertainty [17].

Note 5: ISO/REMCO has an analogous definition [17] but uses the modifiers "metrological" and "metrologically" to refer to both quantity and nominal property.

It should be mentioned here that definitions of RM and CRM have undergone symptomatic evolution since the first ISO/REMCO publication [18] and this is probably not the end of the activity of international bodies in this domain. In the CRM definition given in VIM2 [19] the requirement of traceability described as "property of a measurement result relating the result to a stated metrological reference through an unbroken chain of calibrations of a measuring system or comparisons each contributing to the stated measurement uncertainty," could suggest, if rigorously treating this requirement, that at this moment practically no solid CRM for inorganic trace analysis exists. These RMs were usually certified with the use of analytical techniques that require sample dissolution, which could be interpreted as breaking the chain of comparisons. It is no wonder that these definitions, being the product of orthodox metrologists who apparently did not appreciate the unique characteristics of chemical measurements, had to be revised and tempered. In VIM3 "metrological traceability chain" is defined as the "sequence of measurement standards and calibrations that is used to relate a measurement result to a reference" [1]. The method by which traceability should be realized through the use of CRMs is still being discussed [20]. In reality, despite common acknowledgement of the importance of traceability of the certified values, there is no universal consensus about assuring traceability in practice and its documentation [20]. Three major European CRM producers, the Institute for Reference Materials and Methods (IRMM), Bundesanstalt für Materialforschung und Prüfung (BAM), and LGC Standards, recently established cooperation on CRM production and accepted common principles concerning preparation and certification of RMs and how to document traceability in the certificates [21].

One can easily note that, as the years went by, the definitions of CRM became longer and longer as more and more detailed comments were added. This is a result of the efforts to accommodate many different properties in one definition. For example, "nominal properties" were included in VIM3 for the definition of CRM, and examples of such properties are, among others, "sex of a human being" or "ISO two-letter country code." When the definition becomes too broad, its proper understanding by the wide circle of practical users starts to encounter several problems.

4.5 Kinds of Reference Materials

There are several kinds of RMs [2, 22, 23]:

- RMs for chemical composition (steels, alloys, gases in metals, biological materials of plant and animal origin, geological materials, ores, materials for clinical analysis, glasses, ceramics, etc.)

- RMs for isotopic composition
- RMs for physical properties (ion activity, optical properties, radioactivity, metrology, conductivity, magnetic properties, etc.)
- RMs for engineering and technical properties (standard rubbers, standard sieves, etc.

A special kind of RM are standard gaseous mixtures and silica gels or glasses with the surface modified by chemical operations, which during controlled pyrolysis release definite volatile compounds [23]. There are also RMs prepared on the basis of liquids (e.g., natural waters) [24].

Another classification of RMs takes into account their practical applications:

- CRMs in the area of technical and industrial materials (e.g., pure metals, alloys, catalysts for cars, semiconductors, fossil fuels, glasses, plastics)
- CRMs for medical and biological studies used in health protection, forensic toxicology, biotechnology (e.g., body fluids, preparations representing individual genes)
- CRMs used for the evaluation of purity and composition of substances (solid and liquid organic and inorganic compounds, chemical elements)
- CRMs with certified physical properties such as thermal (thermal conductivity), optical (absorbance, wave length), and mechanical (hardness)
- Matrix-type CRMs for research on food and feed (e.g., potable water and drinks, genetically modified organisms, protein, fat, nitrates, amino acids, fiber).
- CRMs for environmental studies (e.g., water, soil, plants, animal tissues as bioindicators)

The subjects of this chapter are exclusively RMs for chemical composition, intended for inorganic analysis with special emphasis on trace analysis.

It should be remembered [2] that in earlier literature (and even in some recent publications) and in common language, RMs are often called “standards” or both terms are used as equivalent to each other. RMs of the biological or geological type, certified on the basis of ILC, were sometimes named “natural matrix standards.” Echoes of this vocabulary have been preserved, apparently because of an earlier terminological tradition of one of most significant RM producers, the National Institute of Standards and Technology (NIST), formerly the National Bureau of Standards (NBS). CRMs issued by NIST are still designated “standard reference materials” (SRMs). The original definition of SRMs is as follows: “well characterized materials, produced in quantity, that calibrate a measurement system to assure compatibility of measurement in the nation” [25].

Irrespectively of historical aspects, the word “standard” should be reserved, at present, for cases dealing with the highest metrological quality. For instance, very pure metals and high purity compounds with exactly established stoichiometry can be regarded as primary measurement standards and serve for the preparation of standard solutions.

As mentioned in the section “Methods of checking accuracy,” the basic function of CRMs is the transfer of accuracy and assuring compatibility and traceability of

analytical measurements on the global scale. When CRMs are used by laboratories for quality assurance, the results of these laboratories are traceable to SI or other international units. Therefore, the CRM itself must also fulfill rigorous quality criteria. Several ISO Guides (series 30–35 [26–32] exist that describe general rules concerning preparation, certification, and use of CRMs as well as requirements with respect to CRM production. Plurality of kinds and uses of CRMs makes it difficult or impossible to formulate international regulations that would be fulfilled for every conceivable CRM.

ISO Guide 30 with supplements [26, 27] gives most definitions and terms associated with the CRM. ISO Guide 31 [28] describes data that should be given on the certificates and labels. The principles of the use of CRMs are given in ISO Guides 32 and 33 [29, 30]. ISO Guides 34 and 35 concern requirements for CRM producers, in particular the quality assurance system required [31]. The ways of assigning values to quantities, including statistical methods, estimation of uncertainty, and assuring traceability, are dealt with in ISO Guide 35 [32]. In the newest version of ISO Guide 34 [31] there are also requirements referring to realization of traceability in the study of homogeneity, stability, and characteristics of a CRM. ISO Guide 34 [31] together with ISO 17025 norm [33] are the basic documents used in the process of accreditation of CRM producers, in agreement with the resolution of International Laboratory Accreditation Co-operation (ILAC) in 2004. Major European CRM producers, namely IRMM (2004/2005) and LGC (2006), obtained their accreditation this way.

4.6 RM and CRM for Chemical Composition Intended for Inorganic Trace Analysis

Any CRM for chemical composition is suitable for the purpose of trace analysis if it is certified for trace constituents and minimum sample mass, which guarantees that the homogeneity of the material is appropriate for the analytical method used in a laboratory. Taking into account the matrix, it if often useful to distinguish the following main classes of reference materials for chemical composition for inorganic trace analysis:

- Geological RM (e.g., minerals, rocks, ores, soils)
- Biological RM (e.g., animal and plant tissues, body fluids, food and feeding stuffs)
- Environmental RM (e.g., waters, sediments, bioindicators, soils, ashes and dusts)
- Industrial/technical RM (e.g., metals, alloys, structural materials, semiconductors, glass, ceramics)
- Standards solutions and gas mixtures

Table 4.3 presents examples of the main classes of CRMs.

Table 4.3 Examples of CRMs for the purpose of trace analysis

CRM	Category	Producer	Analytes with certified values	Analytes with information values
SRM 688 bazalt rock	Geological	NIST	Al_2O_3, FeO, Fe_2O_3, K_2O, MnO, Na_2O, P_2O_5, SiO_2, TiO_2, Cr, Rb, Sr, Th, Pb	CaO, CO_2, F, MgO, Ce, Co, Eu, Hf, Co, Lu, Sc, Ba, Tb, U, Yb, Cu, Ni, V, Sm, Zn
BCR 464 total and methyl mercury in tuna fish	Biological	IRMM	Total Hg; CH_3Hg^+	
ERM BD150 skimmed milk powder	Biological	IRMM	Ca, Cd, Cl, Cu, Fe, Hg, I, K, Mg, Mn, Na, P, Pb, Se, Zn	
BCR 482 lichen	Biological/ environmental	IRMM	Al, As, Cd, Cr, Cu, Hg, Ni, Pb, Zn	
IAEA-375 soil	Environmental	IAEA	^{106}Ru, ^{125}Sb, ^{129}I, ^{134}Cs, ^{137}Cs, ^{232}Th, ^{40}K, ^{90}Sr, Th	^{228}Th, ^{234}U, ^{238}U, ^{238}Pu, $^{239+240}Pu$, ^{241}Am, As, Ba, La, Ni, Rb
LGC6189 river sediment – extractable metals[a]	Environmental	LGC	As, Cd, Cr, Cu, Mn, Mo, Ni, Pb, Zn	Ba, Se
D 271-1 stainless steel	Industrial	BAM	O, N	
ERM-EB504 car catalyst	Industrial	BAM	Pd, Pt, Rh	
SRM 2614a carbon monoxide in air	Environmental, gas mixture	NIST	CO	

[a]Content of metal species extractable by a prescribed reference procedure

Two kinds of property values are given in the certificate: certified values and information values. The certified values are always quoted together with their uncertainty limits, whereas information values are quoted as numbers without the uncertainties. The information values should serve only to indicate the approximate level of analyte concentration and cannot be used for assessment of the accuracy of the method.

The certificate should contain all information needed by users of the CRMs. The amount of detail included in a certificate depends on the nature of the material and the availability of a certification report. When a report is not supplied with a certificate, the amount of detail in the certificate should be large enough to allow the user make the right decision about whether the intended application of the CRM is correct. As an example, a full description of the adopted preparation and certification approach is included in the certificate of the CRMs for chemical

composition provided by the INCT (Institute of Nuclear Chemistry and Technology, Warsaw, Poland) and can be found on the institute website, www.ichtj.waw.pl/certificates [34].

4.7 Preparation and Certification of RMs for Chemical Composition Intended for Inorganic Trace Analysis

Preparation and certification of RMs is a complicated, multistage, and time-consuming task. Attention to detail at all stages of this task is obligatory because one failure at any stage could shatter previous achievements and make the candidate CRM useless. Correct cooperation of many experienced analysts and constant supervision by the coordinator from the beginning to the end of the whole campaign is necessary for final success.

A general strategy for preparation and certification of matrix CRMs for inorganic trace analysis adopted by INCT is presented in Table 4.4 [35, 36]. Discussion of the large number of details concerning preparation of RMs is beyond the scope of this chapter. Comprehensive information concerning various stages of

Table 4.4 Procedure for preparation and certification of matrix CRMs for inorganic trace analysis, developed and adopted at INCT [35, 36]

Strategy of preparation and certification of CRMS (INCT)
Choice of the type of material
Collection of suitable amount of material
Preparation (comminution, grinding, sieving, isolation of fraction of suitable particle size, homogenization, etc.)
Preliminary stability tests
Selection and purchase of suitable containers; designing and printing of labels
Preliminary homogeneity testing
Characterization of the material with respect to the content of the main constituents (optional)
Determination of particle size distribution
Distribution of the material into containers
Final homogeneity checking
Radiation sterilization (mandatory for biological materials)
Establishment of the method for determination of moisture content
Planning and implementation of short-term and long-term stability testing
Organization of interlaboratory comparison
Statistical evaluation of results (detection and rejection of outliers; calculation of the means, standard deviations, confidence intervals, combined expanded uncertainties, etc.)
Assigning of certified and information values on the basis of previously formulated and thoroughly tested criteria
Printing of the certificate
CRM ready for distribution and sale
Monitoring of long term stability during the whole storage period

handling the candidate CRM, homogeneity checking, establishing the method for moisture content determination, stability testing, radiation sterilization, and organization of ILCs can be found in original publications [9–14, 35–44].

Historically speaking, there have been many different approaches to the process of certification. They can be roughly classified into the following categories:

1. Certification by one organization using a definitive method or at least two independent reference methods (e.g., NIST)
2. Certification based on the results of ILC:
   (a) Select ILC – only the results supplied by a network of select expert laboratories (e.g., BCR)
   (b) Open ILC – comparison with the participation of many volunteer laboratories, such as INCT, IAEA, United States Geological Survey (USGS), Canadian Certified Reference Materials Project (CCRMP), and Groupe International de Travail–International Working Group (GIT-IWG)

There have also been a plethora of ideas on how to elaborate and interpret the results of ILCs.

The INCT has been using an original certification approach based on results of worldwide ILC. The intercomparison data provided by participating laboratories are statistically evaluated by means of an approach based on an outlier rejection procedure, which utilizes concurrently the statistical tests of Dixon, Grubbs, skewness, and kurtosis at the significance level of 0.05, followed by calculation of the overall arithmetic means after outlier rejection, standard deviations, standard errors, and confidence intervals. The performance of this procedure based on outlier rejection was examined on ILC data using test materials of known true values of elemental composition or radionuclide content [45]. The overall means obtained were invariably in good agreement with the true value. In real certification cases, the final decision on whether the overall mean obtains the status of certified or information value, or is out of classification, is made using previously formulated arbitrary criteria. These criteria take into consideration the ratio of the one-sided confidence interval and the overall mean, number of accepted laboratory means and analytical techniques used, and the agreement between results obtained by different analytical techniques [37–39]. The status of certified value is given only when all criteria are met simultaneously. When this is not the case, but the results fulfil slightly less demanding criteria, the status of information value is assigned. The reliability of this method of data evaluation was confirmed, inter alia, by comparing the certified values with their confidence limits [37] for CTA-OTL-1 (oriental tobacco leaves) CRM with the Huber robust means and confidence limits as recommended by ISO [31].

Two auxiliary procedures (requesting participants to analyze a CRM provided by the organizers and the use of definitive methods) were involved in the certification procedure to strengthen quality assurance and traceability of the certified values.

In several recent ILCs, the participating laboratories analyzed a candidate CRM together with an unidentified CRM provided by the organizer. This enabled the creation of two databases. The first contained all results supplied by participating laboratories for all elements in the candidate CRM. The second database was created from the first database for those elements for which certified values in the CRM (sent to participants) were available. This database contained only results from laboratories for which the confidence limits of the laboratory mean for a particular element overlapped with the confidence limits of the CRM. Each of the two databases was subjected to statistical evaluation as described above. Most of the elements were certified from the analysis of the second database, although overall means from both databases were usually very close to each other.

Good agreement of the mean values obtained for CTA-OTL-1 (used as an unknown CRM) in the ILCs conducted in the years 2000–2005 with the certified values (ILC in 1991) confirmed the correctness and reliability of the adopted certification procedure and stability of CRMs prepared according to the INCT procedure [37, 40].

Definitive methods (i.e., ratio primary reference measurement procedures, RPRMPs) based on radiochemical NAA (RNAA) were elaborated for several elements [46–48]. As methods of the highest metrological quality, they served as a tool for independent verification of reliability of the certified values. In all cases where these methods could be used, there was very good agreement between the certified values established by the procedure described above and the results obtained by RPRMPs. Certified values are presented in the form:

$$X \pm U \tag{4.1}$$

where X is the certified value and U is expanded uncertainty ($U = 2u_c$). The combined standard uncertainty, u_c is given by the formula:

$$u_c = \sqrt{u_{\text{interlab}}^2 + u_{\text{lstab}}^2 + u_{\text{inhom}}^2 + u_m^2} \tag{4.2}$$

where u_{interlab} is estimated as the standard deviation of the overall mean, u_{lstab} the standard uncertainty estimated from long-term stability studies, u_{inhom} the standard uncertainty estimated from homogeneity studies, and u_m the standard uncertainty as a result of moisture determination. Sometimes the short-term stability u_{shstab} is also taken into account in the right hand part of Eq. (4.2) but usually it is negligible and can be neglected.

Certified values are always quoted together with their uncertainties. Information values are quoted as numbers only. Examples of the certificates for CRMs issued by INCT can be found at the website www.ichtj.waw.pl.

4.8 Features of a Good CRM

The number of CRMs available on the market is, and probably always will be, smaller than the actual need of the scientific community at large. This does not mean, however, that the analyst in pursuit of quality assurance can take any CRM that is at that moment on a shelf in the laboratory. Five general criteria can be formulated to characterize basic features of a CRM [15, 35] that is fit for a given type of analysis:

- Certified for possibly a great number of trace elements

  Contemporary methods of trace analysis such as NAA, ICP-OES, and ICP-MS make possible simultaneous determination of several elements. Determination of only one or two elements is relatively rarely, requested. Therefore the CRM certified for many trace elements helps to save time and money.
- Good homogeneity with respect to trace elements, including those present at very low concentrations

  Homogeneity defines the degree to which a property or substance is randomly distributed throughout a given material [49]. In the language of analytical chemistry, it means that samples of a given mass taken from this material have the same average composition within the appropriate confidence limits [50]. A similar definition [32] states that a material is perfectly homogeneous with respect to a property if there is no difference in the values of this property in various parts of the material. In practice, it is assumed that a material can be considered homogeneous when the differences in values of a property in various parts of the material can be neglected in comparison with the component of uncertainty stemming, for example, from the method of determining this property.

It should be remembered that solid CRMs with a natural matrix (geological, biological, environmental, etc.) are always inhomogeneous at the microscopic scale because they consist of individual minerals (geological materials) or tissues (biological materials). Apparent homogeneity is achieved by comminution and accurate mixing of the individual particles (grains). Sometimes, to characterize homogeneity (or rather inhomogeneity) of a material, the Ingamells sampling constant, K_s [51, 52] is used:

$$K_s = R_s^2 \times m_s \tag{4.3}$$

where R_s^2 is the sampling variance (in percent) characterizing inhomogeneity with respect to a given analyte in a sample with defined particle size and m_s is the mass of sample taken for analysis. K_s is the sampling constant, expressed in units of mass and equal to the mass of the sample guaranteeing sampling uncertainty of 1 % (at confidence level 68 %).

Some workers have also used $K_s^{½}$ to characterize the homogeneity of materials [51, 53].

It can be shown [35, 41] that for a fictitious case of a two-component mixture in which the component of interest (trace element), A, is present only in grains containing 100 % of this component, the sampling constant is given by:

$$K_s \cong 10^4 \frac{m_{\text{particle}}}{C_A} \tag{4.4}$$

where m_{particle} is the mass of single particle (grain) of the analyzed material and C_A is the concentration of a trace element (g/g).

As can be seen, the chances that a material appears inhomogeneous with respect to a trace component are greater with a lower concentration of this component and greater particle size of the material [35, 50]. Therefore, CRM producers should always state the minimum mass of material that should be taken for analysis. Modern instrumental methods now allow analysis of very small samples; however, one should keep in mind that errors due to inhomogeneity can dramatically increase with a decrease in the effective mass being analyzed, and that CRMs intended for microanalysis are scarce or nonexistent [42, 43].

- Short- and long-term stability

CRMs must be stable throughout the specified time because only then is the certified value together with its uncertainty fully reproducible, provided the storage conditions for the material were appropriate. Therefore, the analyst should pay attention to the shelf life of the material, which should be indicated in the certificate. The requirements for carrying out stability tests and defining the shelf life of a CRM in the certificate are described in the appropriate ISO documents [26–32]. Stability tests are carried out by methods similar to those used in pharmacy. They consist in determination of changes in the values of particular properties of a CRM during storage under the specified conditions [54–58]. The tests usually last 2 years and their results are extrapolated into the future. Generally, both short-term stability in conditions simulating transport and long-term stability in conditions of storage are studied. In the case of CRMs intended for inorganic trace analysis, the changes in concentrations of individual elements are determined as a function of time. The results are interpreted using trend analysis. Similarly to homogeneity study, it is important that the analytical method used in stability tests demonstrates low uncertainty of the results. The lack of statistically significant difference (differences smaller than those corresponding to statistical deviation of repeatability of the measurement method) indicates that the material is sufficiently stable and can be used as a CRM. Concentration changes as a function of time can be then presented in the form of a straight line equation. Standard uncertainty of stability equals the standard deviation of the slope of the straight line [54, 58, 59]. The shelf life of a CRM is especially important if it is used for monitoring the quality of laboratory work with control charts over a long period of time.

- Kind of matrix and concentration of trace elements similar to those in routinely analyzed samples

This requirement is based on an assumption that the possible systematic errors, if any, as a result of matrix effects, spectral interferences, etc. are the same or at least similar in the CRM and in the analyzed samples.

- Easy method for the determination of moisture

Determination of moisture is needed to refer the concentration of elements to the well-defined "dry mass." Certified values in most cases are expressed in mass units per dry mass of the CRM. In reality, the important point is not the exact determination of water in a material, but devising a method of drying that gives reproducible values for conventional dry mass. At the same time, the devised method should be simple and accessible for most common laboratories.

All data important for a CRM should be included in the printed certificate [28].

4.9 Application of CRMs

CRMs play a significant role in quality assurance systems in analytical laboratories, especially in the case of trace analysis. Generally, they are applied in analytical practice to:

- Verification of accuracy, precision, and reliability of the results of analysis
- Development of new analytical procedures
- Validation of analytical methods
- Confirmation of laboratory or analyst performance (competence)
- Comparison of results by different analytical methods
- Internal quality control (e.g., using Shewhart control charts)
- Establishment of measurement traceability
- Calibration of measurement equipment
- Achieving and maintaining accreditation

A commonly recommended and accepted way of checking accuracy is the analysis of CRMs along with routinely analyzed samples. The RM(s) applied for this purpose should obviously be as similar as possible in both the type of matrix and content of determined element(s). In this case, it can be assumed that the possible systematic errors resulting from matrix effects, spectral interferences, etc. are the same or at least similar in the CRM and in the analyzed samples. The result of measurement of $X_m \pm U_m$ can be then compared with the certified value $X_{ref} \pm U_{ref}$, where U_m and U_{ref} are expanded uncertainties of the measurement result and certified value, respectively. Good agreement of the result of measurement and the certified value confirms correctness of analysis. Taking into account numerical values, the following condition should be fulfilled [60]:

$$|X_m - X_{ref}| \leq 2\sqrt{u_m^2 + u_{ref}^2} \tag{4.5}$$

where u_m and u_{ref} are standard uncertainties of measurement result and certified value ($k = 1$), respectively.

The numerical value of $u_\Delta = \sqrt{u_m^2 + u_{ref}^2}$ should be used to estimate the component of uncertainty associated with determination of bias (i.e., uncertainty of the statement that the bias is not significant compared with the combined uncertainty and can therefore be neglected). [61].

Example

Result of total Se determination $X \pm U$ ($k = 2$) in NBS 1547 peach leaves by RNAA is 127 ± 5 ng/g [62], and the certified value for Se is 120 ± 10 ng/g. Therefore, the standard uncertainties are $u_m = 5/2 = 2.5$ ng/g, $u_{ref} = 10/2 = 5$ ng/g, $u_\Delta = \sqrt{2.5^2 + 5^2} = 5.59$, and $U_\Delta = 2u_\Delta = 11.2$. The numerical value of $|X_m - X_{ref}| = |127120| = 7$ is less than U_Δ. Thus, both values are not significantly different and the method can be regarded as free from systematic error.

Normally, CRMs are used for the verification of accuracy, precision, and reliability of the results of analysis carried out in a laboratory (i.e., for checking the quality of its routine work). The CRM is analyzed at specific intervals and the results obtained are used to draw control charts (e.g., Shewhart chart) [63]. This allows visual assessment of the measurement system, the emergence of systematic errors, etc. Application of CRMs for the construction of control charts is advantagous because of the homogeneity and stability of CRMs, and the ability to assess the accuracy of the results obtained in the laboratory by comparison with the certified value.

Demonstrating that the analytical method used is accurate is equivalent to showing that the stages of sample preparation (dissolution, extraction etc.), which can break the chain of traceability, do not cause systematic error. The results of the measurements are then traceable to the units in which the certified values are expressed.

CRMs are also used for calibration of the measurement instrument. For example, pure metals or alloys are used for X-ray fluorescence (XRF) and other spectroscopic methods in industrial laboratories. Because the uncertainty u_{ref} associated with a certified value contributes to the total uncertainty of the measurement, u_{ref} should be as small as possible. Therefore, wherever possible, CRMs used to calibrate instruments should be pure substances or solutions. Matrix CRMs are not recommended for calibration of measuring instruments, because the uncertainty of certified values are generally higher than those of pure substances or solutions. Moreover, using this approach, an analyst is deprived of independent verification of the accuracy of obtained results, which should be the primary function of CRMs.

The use of CRMs is one of the requirements of ISO/IEC 17025: 2005 (paragraph 5.6.3) [33], and is therefore needed to obtain and maintain accreditation by the laboratory. CRMs are also used as test materials in ILCs. However, this is not common practice because it causes a substantial increase in comparison costs.

4.10 Availability of CRMs

Information about CRMs is available from a number of sources. Many suppliers provide a database on their website. The recognized producers of CRMs in Europe are IRMM, LGC, and BAM. Information about CRMs produced by them, called European Reference Materials (ERMs). can be found on the ERM website (www.erm-crm.org). The ERMs are divided into six different categories (groups):

- Industrial and engineering materials certified for composition
- Health-related matrix materials certified for composition
- Materials certified for physical properties
- Food/agriculture and related matrix materials certified for composition
- Non-matrix materials certified for purity and concentration or activity
- Environmental and related matrix materials certified for composition

Figure 4.2 shows the percentage of CRMs in the various categories.

INCT, a Polish producer of CRMs for inorganic trace analysis has issued ten CRMs (Table 4.5). Details of these CRMs can be found on the producer's website, www.ichtj.waw.pl

The leading CRM producers in the world are NIST, the National Research Council of Canada (NRCC), and the International Atomic Energy Agency (IAEA). Information about CRMs produced by NIST can be found at the NIST website, www.nist.gov/srm/index.cfm

IAEA currently specializes in the production of certified RM for radioactive isotope content, but also offers CRM for inorganic trace analysis, organic analysis, and determination of stable isotopes. Information can be found via the IAEA homepage, www.iaea.org, or directly from nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/index.htm

ISO-REMCO established the COMAR database in 1984 [64]. Currently, the COMAR database includes information about more than 10,000 RMs/CRMs from over 200 producers in more than 20 countries [65].

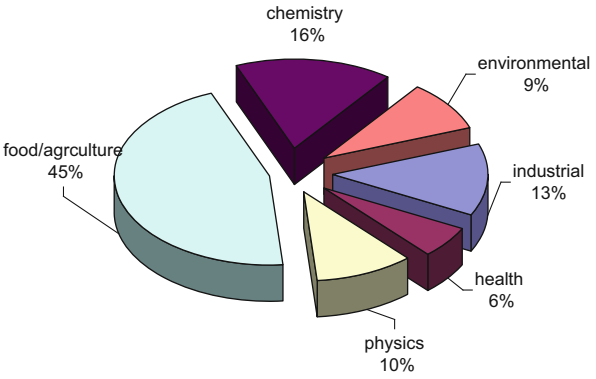

Fig. 4.2 Different classes of certified reference materials

Table 4.5 CRMs produced by INCT

CRM	Elements with certified value	Elements with information values
CTA-AC-1 apatite concentrate	Ba, Ca, Ce, Co, Cu, Eu, Gd, Hf, La, Lu, Mn, Na, Nd, Sc, Si, Sm, Ta, Tb, Th, Ti, U, V, Y, Yb, Zn	Al, Cr, Dy, Er, Fe, Ho, K, Mg, Ni, Pr, Sr, Zr
CTA-FFA-1 fine fly ash	Al, As, Ba, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, F, Fe, Gd, Hf, La, Li, Lu, Mn, Na, Nd, Ni, P, Pb, Rb, Sb, Sc, Si, Sm, Sr, Ta, Tb, Th, Tm, U, V, W, Y, Yb, Zn	Be, Ca, Cd, Ga, In, K, Mg, Mo, Se, Ti
CTA-OTL-1 oriental tobacco leaves	Al, As, Ba, Br, Ca, Cd, Ce, Co, Cr, Cs, Cu, Eu, K, La, Li, Mg, Mn, Ni, P, Pb, Rb, S, Se, Sm, Sr, Tb, Th, V, Zn	Au, Cl, Fe, Hf, Hg, Mo, Na, Sb, Sc, U, Yb
CTA-VTL-2 Virginia tobacco leaves	As, Ba, Br, Ca, Cd, Ce, Cl, Co, Cr, Cs, Cu, Fe, Hf, Hg, K, La, Li, Mg, Mn, Mo, Ni, P, Pb, Rb, Sb, Sm, Sr, Tb, Th, U, V, W, Zn	Al, Eu, Na, S, Sc, Se, Si, Ta, Ti, Yb
INCT-TL-1 tea leaves	Al, As, Ba, Br, Ca, Cd, Ce, Cl, Co, Cr, Cs, Cu, Eu, Hg, K, La, Lu, Mg, Mn, Na, Ni, Pb, Rb, S, Sc, Sm, Sr, Tb, Th, Tl, V, Yb, Zn	B, Fe, Hf, Nd, P, Sb, Se, Ta, Ti, Tm
INCT-MPH-2 mixed Polish herbs	Al, As, Ba, Br, Ca, Cd, Ce, Cl, Co, Cr, Cs, Cu, Eu, Hf, Hg, K, La, Lu, Mg, Mn, Nd, Ni, Pb, Rb, S, Sb, Sc, Sm, Sr, Ta, Tb, Th, V, Yb, Zn	Fe, Mo, Na, P, Ti, Tl, U, W
INCT-CF-3 corn flour	B, Br, Cl, Cu, Fe, K, La, Mg, Mn, Mo, Ni, P, Rb, S, Sc, Zn	Al, As, Ba, Ca, Cd, Co, Cr, Cs, Hg, Na, Pb, Sb, Sr, Ti
INCT-SBF-4 soya bean flour	Al, B, Ba, Br, Ca, Cl, Co, Cs, Cu, Fe, K, La, Mg, Mn, Mo, Ni, P, Rb, S, Sr, Th, Zn	Cd, Cr, Hg, Na, Pb, Sc, Sm, Ti, V
INCT-OBTL-5 basma oriental tobacco leaves	Ag, Al, As, B, Ba, Br, Ca, Cd, Ce, Co, Cs, Cu, Er, Eu, Hf, Hg, K, La, Mg, Mn, Mo, Nd, Ni, P, Pb, Rb, S, Sb, Sc, Sm, Sr, Ta, Tb, Th, V, Yb, Zn	Au, Be, Cl, Cr, Dy, Fe, Gd, Ho, Li, Lu, Na, Pr, Ti, Tl, Tm, U, Y
INCT-PVTL-6 Polish Virginia tobacco leaves	Ag, Al, As, B, Ba, Br, Ca, Cd, Ce, Co, Cu, Er, Eu, Hg, Hf, K, La, Li, Mg, Mn, Mo, Nd, Ni, P, Pb, Rb, S, Sb, Sc, Sm, Sr, Ta, Tb, Th, V, Zn	Bi, Cl, Cs, Fe, Na, Pr, Sn, Ti, Tl, U, Y, Yb

Information on CRMs produced by IRMM can be found on the producer's website, ec.europa.eu/jrc/en/reference-materials, and on those produced by BAM via www.bam.de/en/fachthemen/referenzmaterialien/index.htm.

The Virtual Institute for Reference Materials (VIRM) offers via its homepage (www.VIRM.net) a wide range of tools for quality assurance and quality control, a list of reference material producers, and a large database of CRMs, newsletters, etc. Access to the CRM database is free, and access to other services offered by VIRM requires a subscription fee.

Information on available CRMs and advice on their choosing can be also obtained from LGC Standards via its homepage, www.lgcstandards.com.

4.11 Conclusions

CRMs are an important tool for quality assurance and quality control in analytical laboratories. They are used for verification of accuracy and precision (i.e., reliability of the results of analysis, validation of analytical procedures, establishing measurement traceability, and calibration of measurement equipment). Use of CRMs is recommended by the ISO/IEC 17025 standard and therefore it is obligatory for those who wish to obtain and maintain accreditation of the laboratory.

CRMs are generally expensive as a result of the laborious and time-consuming preparation procedures. Thus, their selection for individual use should be carefully considered. It should be kept in mind that a CRM should be as similar as possible in both the type of matrix and the content of determined element(s) to the test samples. Only then can it be expected that conclusions regarding the accuracy of the obtained results, drawn on the basis of CRM analysis, are fully reliable.

Attention should be also paid to the information contained in the certificate about preparation of the material, adopted method of certification, certified values and their associated uncertainties, homogeneity and stability of the material, method of moisture determination, statements on traceability, expiry date, and instructions on how to proceed with the material.

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Chapter 5
Sample Decomposition Techniques in Inorganic Trace Elemental Analysis

Henryk Matusiewicz

5.1 Introduction

Many modern instrumental techniques require the conversion of solid (or solid-containing) samples to solutions before analysis. The terms "decomposition," "destruction," "digestion," "acid-digestion," "dissolution," "ashing," "wet ashing," "oxidative acid digestion," and "mineralization" all refer to this process. In this contribution, the general expression used is "decomposition," which is specified to dry or wet ashing. "Mineralization" refers to those procedures that result in inorganic chemical forms of the analyte only. A variety of techniques can be used for mineralization, from ambient-pressure wet digestion in a beaker on a hot plate to specialized high-pressure microwave heating. Traditionally, decomposition of a sample in elemental analysis requires it to be mineralized in order to remove the organic content. Sample decomposition for total element determination therefore seems to be the recommended procedure on every occasion.

In general, a decomposition procedure is required to alter the original chemical environment of a sample into a digest (i.e., a solution in which the analyte is distributed homogeneously). More specific conditions for a decomposition technique include the following:

(a) Decomposition must be complete. Inorganic materials should be converted completely into soluble compounds, and organic material must be totally mineralized.
(b) Residual matrix components that interfere in detection must be removed. Residues should be quantitatively soluble in a small volume of high-purity acid.

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(c) The decomposition procedure should be as simple as possible and not require complicated apparatuses.
(d) Decomposition should be adapted in an optimal manner to the entire analytical procedure, allowing the possibility of adjustment of the oxidation state of the analyte and, consequently, compatibility with postdecomposition chemistry.
(e) Preference should be given to procedures in which decomposition and separation are achieved in one step.
(f) To minimize systematic errors in the decomposition procedure (contamination, loss of elements, incomplete decomposition) it is necessary to use clean vessels made of an inert material and the smallest amounts of high-purity reagents; dust should be excluded. Reaction chambers should be as small as possible. Precautions should be taken to minimize analyte (element) loss as a result of adsorption on the vessel material (reaction with the vessel material) or volatilization.
(g) Execution should not be hazardous or dangerous for laboratory personnel.
(h) The yield from the decomposition step should be checked using radioactive tracers.

This chapter gives an overview of decomposition methods and recent developments and applications of the decomposition of different materials. Other sample preparation methods, such as chemical extraction and leaching, solubilization with bases, enzymatic decomposition, thermal decomposition, and anodic oxidation, are beyond the scope of this contribution and are not discussed.

5.2 Bibliography

There are numerous publications giving useful information on the decomposition of many conceivable combinations of matrix and analyte. Some comprehensive books and review articles contain material pertinent to either organic [1–4] or inorganic [5–10] matrices; others, to both [11–21].

Within the scope of this chapter, a comprehensive discussion of decomposition techniques is not feasible. For more comprehensive information, various reviews and books are available. Books by Šulcek and Povondra [8], Bock [11], and Krakovská and Kuss [17] are dedicated solely to decomposition methods. Other books deal exclusively with a single technique, microwave-assisted sample preparation [22, 23]; this topic has also been reviewed elsewhere [24–34]. Recommended guidelines for sample preparation (methods of digestion) of different matrices are also available from the *Encyclopedia of Analytical Chemistry* [35]. Although it is difficult to refer to every paper published in this area, the reference list of this chapter gives comprehensive coverage of advances in this topic to date, potential applications, novel developments, and progress in decomposition techniques.

5.3 Sample Decomposition Techniques

Table 5.1 gives an overview of the different decomposition methods for organic and inorganic sample material. The intent is not to present the procedural details for the various sample matrices, but rather to highlight those methods that are unique to each technique and sample.

Table 5.1 Scheme of decomposition methods

Decomposition technique	Required reagents	Application
Wet chemical decomposition		
In open systems		
• Acid digestion (thermally convective wet decomposition) • Microwave-assisted wet decomposition • Ultraviolet decomposition (photolysis) • Ultrasound-assisted acid decomposition	• HNO_3, HCl, HF, H_2SO_4, $HClO_4$ • HNO_3, HCl, HF, H_2SO_4, $HClO_4$, H_2O_2 • H_2O_2, $K_2S_2O_8$, HNO_3, O_3 • H_2O_2, HNO_3	• Inorganic/organic • Inorganic/organic • Waters, slurries • Inorganic
In closed systems		
• With conventional heating (thermally convective pressure digestion) • With microwave heating	• HNO_3, HCl, HF, H_2O_2 • HNO_3, HCl, HF, H_2O_2	• Inorganic/organic • Inorganic/organic
In flow systems		
• With conventional heating • With microwave heating • Ultraviolet decomposition	• HNO_3, H_2SO_4, H_2O_2, HCl • HNO_3, H_2SO_4, H_2O_2, HCl • H_2O_2, $K_2S_2O_8$, HNO_3	• Inorganic/organic • Inorganic/organic • Waters, slurries?
Vapor-phase acid digestion		
• With conventional heating • With microwave heating	• HNO_3, HCl, HF, H_2O_2 • HNO_3, HCl, HF, H_2O_2	• Inorganic/organic • Inorganic/organic
Combustion		
In open systems		
• Dry ashing • Low temperature ashing (combustion in a stream of oxygen) • Cool plasma ashing (Wickbold combustion)		• Inorganic/organic • Organic • Organic
In closed systems		
• Oxygen flask combustion (Schöniger flask) • Oxygen bomb combustion • Combustion in a dynamic system (Trace-O-Mat)		• Organic • Organic • Organic
Fusion decomposition	Fluxes	• Inorganic

5.3.1 Wet Chemical Decomposition

One of the oldest and still most frequently used techniques is wet decomposition in open systems. Wet decomposition can also be used in connection with closed systems. Sample wet decomposition is a method of converting the components of a matrix into simple chemical forms. This decomposition is produced by supplying energy, such as heat; by using a chemical reagent, such as an acid; or by a combination of the two methods. When a reagent is used, its nature is chosen according to that of the matrix. The amount of reagent used is dictated by the sample size, which, in turn, depends on the sensitivity of the method of determination. However, the process of putting a material into solution is often the most crucial step of the analytical process because there are many sources of potential errors (i.e., partial decomposition of the analytes present or some type of contamination from the vessels used). It is beyond the scope of this contribution to discuss all possible systematic errors; further details on how to avoid systematic errors during sample decomposition are discussed in chapter 2 of [36].

The majority of wet decomposition methods involve the use of some combination of oxidizing acids (HNO_3, hot concentrated $HClO_4$, hot concentrated H_2SO_4), nonoxidizing acids (HCl, HF, H_3PO_4, dilute H_2SO_4, dilute $HClO_4$), and hydrogen peroxide. All these acids are corrosive, especially when hot and concentrated, and should be handled with caution to avoid injury and accidents. Concentrated acids with the requisite high degree of purity are available commercially, but they can be purified further by sub-boiling distillation [37].

Wet digestion has the advantage of being effective on both inorganic and organic materials. It often destroys or removes the sample matrix, thus helping to reduce or eliminate some types of interference. The physical properties of common mineral acids used in sample preparation are summarized in Table 5.2.

Most wet decomposition procedures are conducted under conditions that, in terms of temperature or reagents used, must be considered as extreme. Thus, the material of which the flasks, crucibles, and other tools are made must be chosen carefully according to the particular procedure to be applied. The material from which the digestion device is fabricated is also a frequent source of elevated blanks. Elements can either be dissolved from the material or be desorbed from the surface. The nature of the material is very important in this respect. The suitability of materials can be estimated according to the following criteria: heat resistance and conductance, mechanical strength, resistance to acids and alkalis, surface properties, reactivity, and contamination. The specific characteristics of organic and inorganic materials must also be given special consideration. Table 5.3 shows preferred materials for decomposition vessels. The container material in contact with the sample during decomposition also frequently causes systematic errors. Elements can be either dissolved from the material or desorbed from or adsorbed onto the container surfaces. The amount depends on the material, contact time, and temperature. Table 5.4 summarizes the inorganic impurities likely to be encountered with various vessel materials. Borosilicate glass, which contains several

Table 5.2 Physical properties of common mineral acids and oxidizing agents used for wet decomposition

Compound	Formula	Molecular weight	Concentration		Density (kg/L)	Boiling point (°C)	Comments
			w/w (%)	Molarity			
Nitric acid (V)	HNO_3	63.01	68	16	1.42	122	68 % HNO_3, azeotrope
Hydrochloric acid	HCl	36.46	36	12	1.19	110	20.4 % HCl, azeotrope
Hydrofluoric acid	HF	20.01	48	29	1.16	112	38.3 % HF, azeotrope
Perchloric acid (VII)	$HClO_4$	100.46	70	12	1.67	203	72.4 % $HClO_4$, azeotrope
Sulfuric acid (VI)	H_2SO_4	98.08	98	18	1.84	338	98.3 % H_2SO_4
Phosphoric acid	H_3PO_4	98.00	85	15	1.71	213	Decomposes to HPO_3
Hydrogen peroxide	H_2O_2	34.01	30	10	1.12	106	

Table 5.3 Preferred materials for wet decomposition vessels

Material	Chemical name	Working temperature (°C)	Heat deflection temperature (°C)	Water absorption (%)	Comments
Borosilicate glass	SiO_2[a], B_2O_3[b]	<800[c]			Ordinary laboratory glass is not suitable for use in wet decomposition procedures
Quartz	SiO_2[d]	<1200			For all procedures involving wet decomposition of organic material, quartz is the most suitable material for vessels
Glassy carbon	Graphite	<500			Glassy carbon is used in the form of crucibles and dishes for alkaline melts and as receptacles for wet decomposition procedures
PE	Polyethylene	<60			
PP	Polypropylene	<130	107	<0.02	
PTFE	Polytetrafluorethylene	<250	150	<0.03	PTFE is generally used only for decomposition vessels in pressure decomposition systems
PFA	Perfluoralkoxy	<240	166	<0.03	
FEP	Tetrafluorperethylene	<200	158	<0.01	
TFM	Tetrafluormetoxil			<0.01	

[a]SiO_2 content between 81 and 96 %
[b]B_2O_3 content between 3 and 5 %
[c]Softens at a temperature of 800 °C
[d]SiO_2 99.8 %

Table 5.4 Inorganic impurities in selected vessel materials

Element	Borosilicate glass	Quartz	Polyethylene	PTFE Teflon[a]	Glassy carbon
Al	Main[b]	100–50,000	100–3000		6000
As	500–22,000	0.1–80			50
B	Main	10–100			100
Ca	10^6	100–3000	200–2000		80,000
Cd	1000	0.4–10			10
Co	100	1	0.5	2	2
Cr	3000	3–5	20–300	30	80
Cu	1000	10–70		20	200
Fe	2×10^5	200–800	1000–6000	10–30	2000
Hg		1		10^c	1
Mg	6×10^8	10	100–2000		100
Mn	6000	10			100
Na	Main	10–1000	200–10,000	25,000	350
Ni	2000				500
Pb	3000–50,000		200		400
S	Main	Main			85,000
Sb	8000	1–2		0.4	10
Ti	3000	100–800			12,000
Zn	3000	50–100	100	10	300

Data in nanograms per gram (ng/g)
[a]Teflon is a registered trademark of DuPont
[b]Main stands for significant—the concentration of that element is high but without specific values
[c]Strongly dependent on storage conditions

major, minor, and trace elements in relatively high concentrations, is not usually suitable for element determinations in the extreme trace range. Quartz can be considered a pure material and is easily available in varying degrees of purity. For most sample preparation steps in trace (metal) analysis, high-purity quartz is the preferred container (and tool) material. Alternatively, high-purity synthetic polymers can be used in many decomposition applications; examples are polyethylene (PE), polypropylene (PP), polytetrafluorethylene (PTFE), and several fluoropolymers (PFA, FEP, TFM). The apparatus and containers that are used for wet decomposition procedures must be scrupulously cleaned and tested for any possible contamination. Usually, it is sufficient to boil the flasks in concentrated nitric acid, followed by rinsing several times with ultrapure water before use. In cases where this procedure is not adequate, one of the most powerful cleaning procedures is steaming the vessels with nitric or hydrochloric acid in a microwave-heated sealed Teflon vessel [38]. This procedure is particularly recommended for quartz, borosilicate glass, and PTFE vessels.

To summarize this section, nitric acid is an almost universal decomposition reagent and the most widely used primary oxidant for the decomposition of organic matter, because it does not interfere with most determinations and is available commercially with sufficient purity. Hydrogen peroxide and hydrochloric acid can be usefully employed in conjunction with nitric acid as a means of improving the

quality of a decomposition. Hydrochloric acid and sulfuric acid can interfere with the determination of stable compounds. Mixtures with hydrochloric acid are generally used for samples containing principally inorganic matrices, and combinations with hydrofluoric acid are used to decompose silicates insoluble in the other acids. Safety considerations are particularly important when using perchloric acid.

5.3.1.1 Wet Decomposition in Open Systems

Open vessel acid digestion, one of the oldest techniques, is undoubtedly the most common method of sample decomposition or dissolution of organic and inorganic sample material used in chemical laboratories. This very inexpensive technique is of inestimable value for routine analysis because it can easily be automated; all the relevant parameters (time, temperature, introduction of decomposition reagents) lend themselves to straightforward control.

The main advantage of wet decomposition over dry ashing is its speed. However, systems of this type are limited by a low maximum decomposition temperature, which cannot exceed the ambient-pressure boiling point of the corresponding acid or acid mixture. For instance, the oxidizing power of nitric acid with respect to many matrices is insufficient at such low temperatures (boiling point 122 °C). One possible remedy is the addition of sulfuric acid, which significantly increases the temperature of a decomposition solution. Whether this expedient is practical depends on the matrix and the determination method. High-fat and high-protein samples are generally not subject to complete decomposition at atmospheric pressure. Other disadvantages relate to the risk of contamination through laboratory air, the necessarily large amounts of required reagents, and the danger of loss of trace elements. Losses can be kept low by using an excess of acid combined with a reflux condenser and by optimization of temperature and duration. Nevertheless, systems operated at atmospheric pressure are preferred from the viewpoint of workplace safety.

Conventional Heating (Thermally Convective Wet Decomposition)

The conventional approach to wet decomposition entails a system equipped with a heated conventional source (Bunsen burner, heating plate, sand bath, etc.) operating either at a fixed temperature or in response to a temperature program. Acid decompositions are often accomplished in any vessel, usually in glass or PTFE (beaker, conical flask, etc.) with or without a refluxing condenser. However, when a sample is decomposed in open wet digestion, refluxing is compulsory. The necessary apparatus has been described by Bethge [39]. Open block decomposition systems were popular in sample analysis in past decades, but have consistently suffered from the major drawback of their sensitivity to corrosion and subsequent risk of contamination (Fig. 5.1). Therefore, block digestion systems (hotplate techniques) are not considered state-of-the-art technology in trace and ultratrace

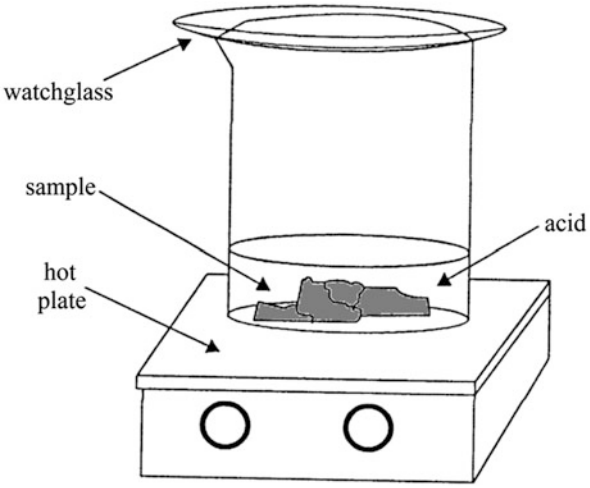

Fig. 5.1 Open digestion

sample preparation. Graphite block digestion systems are becoming more frequently considered. These systems overcome the deficiencies of traditional systems made from stainless steel or aluminum, because the block is manufactured from graphite and typically coated with a fluoropolymer to prevent the possibility of metallic contamination from the surface of the system during handling of the samples. Graphite block systems present an alternative to the current mainstream technology of open- and closed-vessel digestion systems, as they allow large numbers of samples to be digested simultaneously, thus overcoming one of the major weaknesses of closed-vessel systems. Commonly employed decomposition agents include nitric acid, sulfuric acid, hydrofluoric acid, perchloric acid, and hydrogen peroxide, as well as various combinations of these. Most applications of wet decomposition involve aqueous or organic matrices, such as surface waters, waste water, biological and clinical samples, food samples, as well as soil, sediment and sewage sludge, coal, high-purity materials, and various technical materials. More recently, open systems have progressed; decomposition ramps now usually consist of several vessels equipped with reflux condensers to limit possible volatilization loss of some analytes and to avoid the evaporation of the reactive mixture. Such an assembly is entirely satisfactory for ensuring concurrent digestion of large series of samples. The modern, commercially available Hach Digesdahl Digestion Apparatus (Hach Company, USA) is designed to digest organic and mineral samples for subsequent analysis.

Microwave Heating (Microwave-Assisted Wet Decomposition)

The most innovative source of energy for wet decomposition procedures is microwaves. Because the heating takes place inside the decomposition mixture, microwave-assisted decomposition is more efficient than decomposition using

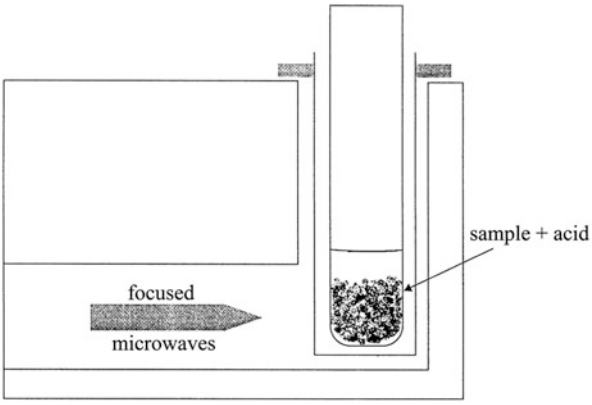

Fig. 5.2 Microwave-assisted open wet digestion

conventional means of heating. Using microwaves, both the speed and efficiency of decomposition for some types of samples considered difficult to solubilize are often improved. Additionally, automation becomes possible with some instrumentation.

Since Abu-Samra et al. [40] reported the application of microwave techniques to wet decomposition of biological samples, there has been rapid development in microwave-assisted decomposition for elemental analysis. Recent reviews [15–36] detail the application of microwave-assisted decomposition to a wide variety of sample types, such as geological, biological, clinical, botanical, food, environmental, sludge, coal and ash, metallic, synthetic, and mixed, and present specific experimental conditions as a function of the matrix to be digested. The earliest attempts at microwave-assisted digestion were performed using domestic microwave ovens (Fig. 5.2). This was necessary because commercial devices were not available at the time. The use of domestic microwave ovens in laboratory experiments should be discouraged because of safety and performance. Microwave-assisted decomposition in open systems at atmospheric pressure is generally applicable only with simple matrices or for strictly defined objectives, and the results are reproducible only if the specified decomposition parameters are strictly observed. The performance of focused-microwave-assisted systems and a wealth of applications have been reviewed by White [24] and Mermet [25] and very recently by Nóbrega et al. [41]. Focused-microwave-assisted sample preparation is a suitable strategy for dealing with high masses of organic samples (up to 10 g). Losses may be encountered with mercury and possibly also with organometallic compounds. Addition of sulfuric acid is essential in order to achieve a sufficiently high decomposition temperature using atmospheric pressure equipment, where the boiling point of the acid establishes the maximum decomposition temperature. However, it is important to remember that the presence of sulfate interferes with many procedures for metal determination. Although non-pressurized microwave systems are limited by a low maximum digestion temperature, which cannot exceed the ambient-pressure boiling point of the acid (or the acid mixture), they provide the best option with regard to the safety of personnel because no overpressure can

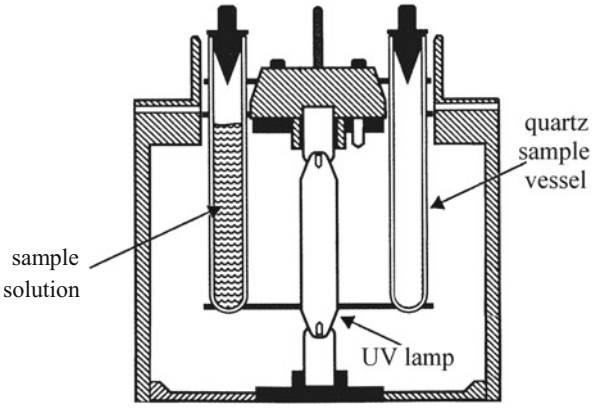

Fig. 5.3 UV irradiation system

occur. Moreover, non-pressurized microwave-assisted digestion is suitable for on-line decompositions in continuous-flow systems.

Very recently, Matusiewicz [42] presented an overview of the different microwave-based systems used for solid and liquid sample pretreatment. It includes relevant publications relating to current research, the unique instrumental approach, and the various commercially available systems and their operating parameters and accessories.

Ultraviolet Decomposition (Photolysis)

Ultraviolet (UV) decomposition is utilized mainly in conjunction with uncontaminated or slightly contaminated natural water matrices (aqueous solutions) such as sea, surface, fresh, river, lake, ground, estuarine, and costal water. Liquids or slurries of solids are decomposed by UV radiation (light) in the presence of small amounts of hydrogen peroxide, acids (mainly HNO_3), or peroxodisulfate (i.e., beverages, special industrial waste water, water from sewage treatment plants, soil extracts) [43]. Dissolved organic matter and complexes of the analyte elements are decomposed to yield free metal ions. The corresponding decomposition vessel should be placed in the closest possible proximity to the UV lamp (low or high pressure) to ensure a high photon flux (see Fig. 5.3). In photolysis, the decomposition mechanism can be characterized by the formation of OH^* and O_2^* radicals from both water and hydrogen peroxide, initialized by UV radiation [43]. These reactive radicals are able to oxidize, giving carbon dioxide and water, the organic matter present in simple matrices containing up to about 100 mg L^{-1} of carbon. Complete elimination of the matrix is, of course, possible only with simple matrices or by combining photolysis with other decomposition techniques [44]. The method does not oxidize all organic components possibly present in water; chlorinated phenols, nitrophenols, hexachlorobenzene, and similar compounds are only partly oxidized. Effective cooling of the sample is essential, because losses might

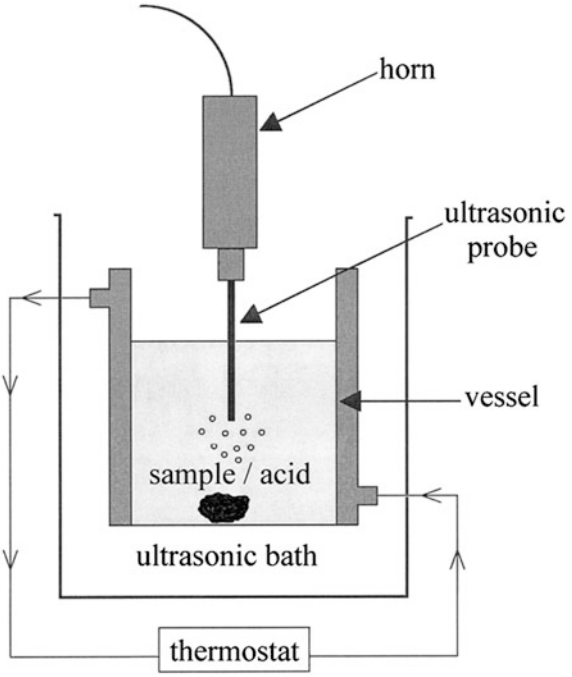

Fig. 5.4 Ultrasound-assisted digestion

otherwise be incurred with highly volatile elements. Hydrogen peroxide addition might need to be repeated several times to produce a clear sample solution. Modern UV decomposition systems are commercially available (see [43] and Table 5.1).

Ultrasound-Assisted Acid Decomposition

Although analytical chemists have shown little interest in the use of ultrasound, its potential usually surpasses that of other conventional auxiliary energies. Thus, ultrasound can be of great help in the pretreatment of solid samples because it facilitates and accelerates steps such as dissolution, fusion, and decomposition. An acid decomposition method that uses an ultrasonic device (bath or probe) has been developed (Fig. 5.4). The propagation of ultrasonic waves characterized by a minimum frequency of 16 kHz results in rapid fluid movement through compression and rarefaction: an enormous number of microscopic cavities are formed, free radicals are generated, chemical layers are dispersed, and contact between the ingredients of the reaction is accelerated. Usually, ultrasonic effects are much more intense in heterogeneous than in homogeneous chemical systems, because emulsification is favored and mass heat transfer in two-phase systems is increased. These effects have been exploited for sample preparation in agriculture, biological, and environmental chemistry [45, 46].

5.3.1.2 Wet Decomposition in Closed Systems

During the last few decades, methods of wet sample preparation using closed vessels have become widely applied. Closed systems offer the advantage that the operation is essentially isolated from the laboratory atmosphere, thereby minimizing contamination. Decomposition of the sample is essentially ensured by a common wet digestion procedure, which is performed under the synergistic effects of elevated temperature and pressure; decomposition occurs at a relatively high temperature as a result of boiling-point elevation. The pressure is, in fact, nothing more than an undesirable (but unavoidable) side effect. These techniques are generally much more efficient than conventional wet decomposition in open systems, the loss of volatile elements is avoided, any contribution to blank values is reduced, and the decomposition of difficult samples is possible. The principal argument in favor of this form of decomposition is the vast amount of relevant experience acquired in recent decades. Closed system decomposition is particularly suitable for trace and ultratrace analysis, especially when the supply of sample is limited.

Because the oxidizing power of a decomposition reagent shows marked dependence on temperature, an arbitrary distinction should be made between low-pressure decomposition and high-pressure decomposition. Low-pressure decomposition (20 bar) is limited to a temperature of ca. 180 °C, whereas with a high-pressure apparatus (70 bar) the decomposition temperature can exceed 300 °C.

Conventionally Heating (Thermally Convective Wet Pressure Decomposition)

The decomposition of inorganic and organic substances in sealed tubes was the method first proposed for pressure digestion at the end of the nineteenth century, and it is still difficult to replace some of these applications by other digestion methods. The use of sealed glass tubes goes back to Mitscherlich [47] and Carius [48], often referred to as the Carius' technique and first described in 1980. Carius undertook digestion of organic materials with concentrated nitric acid at 250–300 °C. The sample and acid were mixed in a strong (thick)-walled quartz ampoule and sealed (Fig. 5.5). The ampoule was transferred to a "bomb canister" and heated in what was called a "bomb oven" for several hours, after which it was cooled, opened, and the contents analyzed. Carius tube decomposition involves the generation of internal pressure in excess of 100 bar at 240 °C. For safety, any stainless steel sleeve jacket (along with solid CO_2 pellets, to maintain equal pressure across the tube wall when heated) that is large enough to contain the Carius tube can suffice as an external pressure vessel [49].

With development of the Carius tube, the field of closed-vessel decomposition was born. Decomposition in autoclaves with metal inner reaction vessels was

Fig. 5.5 Carius tube

originally proposed in 1894 by Jannasch [50], but was not widely employed because of a number of drawbacks (such as strong corrosion of the platinum vessel).

Extensive use of pressure decomposition in analytical procedures began in 1960, as a result of the considerable technological progress in the manufacture of organic polymers. Convectively heated pressure vessel systems have proved to be the most valuable system for guaranteeing complete, or almost complete, digestion of solid samples because they can provide elevated digestion temperatures (200–230 °C) [51]. Most sample vessels for use in thermally convective pressure digestion are constructed from PTFE [52–54], PFA [55], or PVDF [56], although special quartz vessels with PTFE holders [57] or glassy carbon vessels [58] are available for trace analysis purposes. The sample vessel is mounted in a stainless steel pressure autoclave and then heated, usually in a laboratory drying oven, furnace, or heating block, to the desired temperature (Fig. 5.6). Because of the need to examine numerous samples, mechanized multisample pressure digestion systems able to process a large number of samples of the same matrix type have been developed [59]. A cooling circuit can be fitted into the metal casing (jacket) to permit rapid manipulation of the solution formed immediately after removing the digestion bomb from the oven or heating block [60]. Dissolution can be also accelerated by mixing the reactants, preferable using a stirring bar (covered with PTFE) [61]. An alternative design has been proposed by Uchida et al. [62], whereby a small screw cap vial for sample digestion is placed inside a Teflon digestion double vessel. To improve pressure–temperature evaluation and the carbon balance for some materials, a system with a Teflon-lined membrane pressure meter and a thermocouple was designed [63]. Recently, a digestion vessel for use with a convection oven was proposed [64]. The unusual design consists of a vessel with three nested structures:

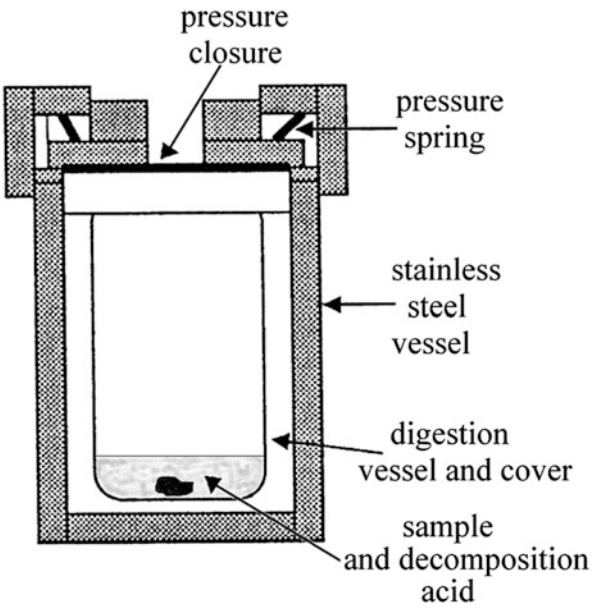

Fig. 5.6 PTFE digestion"bomb"

an innermost PTFE container of 30 mL capacity, an intermediate PTFE container of 100 mL capacity, and an outer stainless steel shell.

All thermally initiated digestions have the disadvantages that a considerable amount of time is consumed in preheating and cooling the digestion solutions and sample vessel [65], the sample size is limited, and it is not possible to visually check the progress of digestion. The contributions of Langmyhr, Bernas, Tölg, and coworkers are worth mentioning with regard to the commercialization of digestion vessels, or digestion bombs as they are often called. Today, there are a number of digestion bombs for a wide market range, including the popular Parr acid digestion bombs (Parr Instrument Company, USA), Uniseal decomposition vessels (Unseal Decomposition Vessels, Israel), stainless steel pressure vessels with Teflon inserts (Berghof Laborprodukte, Germany), the pressure decomposition system CAL 130FEP (Cal Laborgeräte, Germany), and the pressure digestion system PRAWOL (Fleischhacker KG, Germany).

To avoid the problem of loss of mechanical stability at high temperatures, vessels made of quartz are now being used in a new pressure digestion system [66, 67]. The introduction of a high-pressure ashing (HPA) technique by Knapp [68] has not only reduced the effective digestion time but also opened the way to digestion of extremely resistant materials, such as carbon, carbon fibers, mineral oils, etc. A perfected system of wet decomposition under high temperature (320 °C) and pressure (130 bar) developed by Knapp is commercially available as the HPA-S High Pressure Asher system (Anton Paar, Austria).

Very recently, for complete decomposition of organic waste materials, a potent digestion technique was developed [69] based on the prototype of an HPA device

using infrared heating (IR-HP-asher). High-pressure decomposition is conducted in six quartz vessels inside a steel autoclave, with a maximum digestion temperature as high as 300 °C at a pressure of 130 bar. The novelty of this approach lies in the design of the HPA system with IR heating.

Because metal autoclaves are expensive, a pressure vessel without an outer metal casing has been designed. The vessel can be sufficiently well sealed using a screw cap [70]. Volatile components are not lost during heating and the laboratory atmosphere is therefore not contaminated by acid vapors. All-Teflon thick-walled PTFE vessels (bombs) have been used in the dissolution of refractory oceanic suspended matter using HCl, HNO_3, and HF [71]. Translucent Nalgene-sealed bottles have been proposed for wet pressure digestion of biological materials (fish, bird, plant tissue) using a combination of $HClO_4$ and HNO_3 [72]. A method utilizing a pressure digestion technique for real sample matrices using bottles of linear PE has also been proposed [73]. Vessels of PE are transparent, permitting observation of the whole digestion process. Complete decomposition of fatty material with slight overpressure (4 bar) was possible in a closed system completely made from quartz [74]. A closed PTFE bomb (30 mL capacity, screw-cap vessel machined from molded, stress-relieved Teflon-TFE rod) was designed for the digestion of materials using a conventional heating (drying) oven [75].

Microwave Heating (Microwave-Assisted Pressurized Wet Decomposition)

Closed-vessel microwave-assisted decomposition technology has been acknowledged as one of the best solutions for "clean" chemistry applications and has a unique advantage over other closed-vessel technologies in that high temperatures can be reached at relatively low pressures. The vessels used for microwave acid digestion are either low-pressure or high-pressure bombs. The current generation of microwavable-closed vessels are of a two-piece design: liners and caps composed of high-purity Teflon or PFA, with casings (outer jacket) made of polyetherimide and polyetheretherketone or another strong microwave-transparent composite material (Fig. 5.7). The practical working temperature is 260 °C (softening point of Teflon), and their pressure limit is 60–100 bar. Closed-vessel decomposition is ideal for those samples that are being dissolved in HNO_3 and/or HCl.

Microwaves heat the liquid phase, whereas vapors do not absorb microwave energy. The temperature of the vapor phase is therefore lower than the temperature of the liquid phase and vapor condenses on cool vessel walls. As a result, the actual vapor pressure is lower than the predicted vapor pressure. This sort of sustained dynamic, thermal non-equilibrium is a key advantage of microwave technology, because very high temperatures (and, in turn, short digestion times) can be reached at relatively low pressures.

The inspiration for pressure digestion studies came from a US Bureau of Mines report [76], which described how rapid dissolution of some mineral samples could be achieved using a microwave oven to heat samples of an acid mixture contained

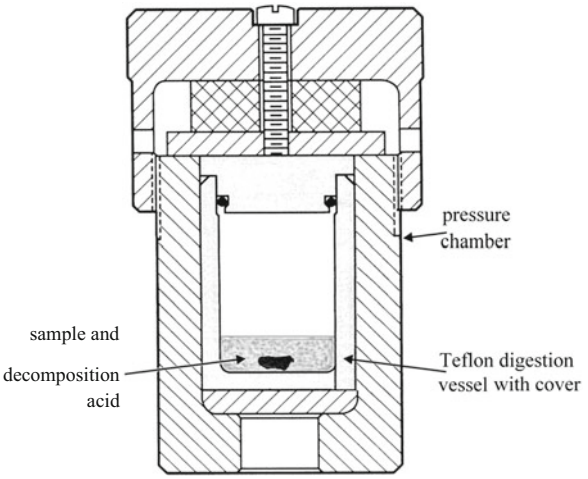

Fig. 5.7 Microwave acid digestion "bomb"

in polycarbonate bottles. Smith et al. [77] substituted Teflon PFA fluorocarbon resin vessels for polycarbonate because of its superior chemical and mechanical properties. Buresch et al. [78] used low pressure-relief containers made of PTFE or quartz. Alvarado et al. [79] exploited modified thick-walled Pyrex glass test tubes fitted with PP screw caps as pressurizable vessels. Kojima et al. [80] modified a Teflon digestion bomb by using a double Teflon vessel with a PP jacket to permit leak-free and safe decomposition of samples. A closed-vessel microwave digestion system was also described [81]. In situ measurement of elevated temperatures and pressures in closed Teflon PFA vessels during acid decomposition of organic samples was demonstrated; temperature and pressure monitoring permitted controlled decomposition and study of decomposition mechanisms.

Laboratory-made all-Teflon bombs, used for low- or medium-pressure work, are also appropriate for microwave-heated digestion [82], especially when fitted with pressure-relief holes, valves, or membranes (rupture discs).

Low-volume microwave-assisted decomposition methods have found application in studies involving small sample sizes, where loss of sample in large digestion equipment is inevitable. Small quantities of tissue (5–100 mg dry weight) are decomposed in high-purity nitric acid by use of a modified Parr microwave acid digestion bomb with modified Teflon liner [83]. The use of low-volume (7 mL) Teflon-PFA closed vessels designed for the digestion of small (100 mg dry mass) samples of biological tissue has been described [84].

In order to prevent excessive pressure rises during closed microwave acid decomposition of fairly large (1 g) samples having high organic content, an open-vessel pre-decomposition technique under reflux was designed to allow the escape of oxidation products, such as carbon dioxide, without incurring evaporation losses of acid or analytes. Following pre-decomposition, the vessels were capped and subjected to microwaves to complete the decomposition under pressure [85].

In an attempt to minimize the delay in opening Teflon pressure vessels following microwave acid digestion, and thus significantly reduce sample preparation time, digestion was carried out with the pressure vessels immersed in liquid nitrogen and using liquid nitrogen as a pre- and post-digestion coolant [86]. In other developments, a special type of Teflon bomb was constructed in which the vapor pressure could be maintained at a moderate level (up to 5 bar) by means of an internal quartz or Teflon cooling spiral. During operation, reflux of the condensed acid and water vapors continuously renew the liquid phase over the sample [87].

Several microwave-heating configurations were presented by Pougnet et al. [88, 89] based on 500 or 1200 W, 2.45 GHz fundamental-mode microwave waveguide cavities, which heat pressure vessels currently used in laboratories for sample decomposition and other applications.

The capsule concept was reviewed in detail by Légère and Salin [90, 91]. The sample is handled in an encapsulated form until it is in the digestion solvent. Operation of the capsule-based microwave-assisted digestion system proceeds in several steps, during which temperature and pressure are monitored.

From the previous discussion, it is clear that microwave acid digestion can be easily adapted for closed-vessel digestions; hence, its application has been limited to digestions in closed Teflon-lined vessels made of non-metallic microwave-transparent materials operating with a maximum upper safe pressure of around 60–100 bar. In response to these limitations, Matusiewicz [92, 93] developed a focused-microwave-heated bomb that could exceed the operational capabilities of existing microwave digestion systems and permit construction of an integrated microwave source/bomb combination, capable of being water or fluid cooled in situ. Another vessel configuration integrates the microwave chamber around the vessel. This consists of one or several microwave-transparent vessels (Teflon, quartz), which can be sealed and enclosed in an acid-resistant stainless steel chamber [94]. The steel chamber acts as both the pressure vessel and microwave chamber. Modern systems can handle acid decompositions at temperatures up to 320 °C and pressures of 130–200 bar.

Very recently, a novel microwave-assisted high-temperature UV digestion system for accelerated decomposition of dissolved organic compounds or slurries was developed [95, 96]. The technique is based on a closed, pressurized, microwave decomposition device wherein UV irradiation is generated by immersed electrodeless Cd discharge lamps (228 nm) operated by the microwave field in the oven cavity. The immersion system enables maximum reaction temperatures of up to 250–280 °C, resulting in a tremendous increase in mineralization efficiency.

Today, there are a number of microwave-digestion bombs and systems available [42].

5.3.1.3 Flow Systems

Discrete vessel systems, whether at elevated or atmospheric pressure, require a large amount of handling. Processes such as assembling, closing, opening, and

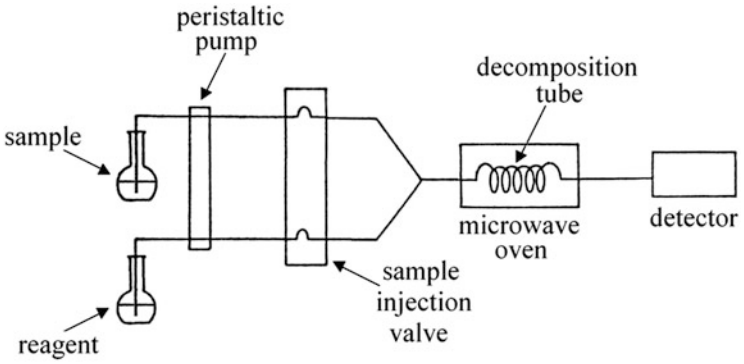

Fig. 5.8 Flow system

positioning the vessel in an ordinary oven or microwave field are laborious and time consuming. Continuous flow-through thermal digestion, UV decomposition, and microwave digestion systems were designed to overcome some of the limitations by replacing the vessels with flow-through tubing (coil). Samples are digested by pumping them through a coil containing a digestion matrix while being heated (thermally, or by UV or microwave) (Fig. 5.8). The continuous flow of a carrier stream through these systems washes the system, removing the need for tedious vessel clean-up procedures. These systems can handle reactions that produce sudden increases in temperature and pressure, or unstable samples. Many different designs for flow digestion systems have been published, but very few meet the prerequisites for high-performance sample decomposition.

Conventional Heating (Thermal)

Many of the disadvantages of sample digestion can be overcome by automating sample preparation in an enclosed system through the use of flow technology.

A well-established digestion system was presented by Gluodenis and Tyson [97]. Here, PTFE tubing is loosely embedded in a resistively heated oven. By using PTFE tubing, the maximum digestion temperature is restricted to ca. 210 °C. The limited mechanical strength of the material allows maximum working pressures of only 35 bar. Therefore, the usual working pressure is about 10–20 bar. The potential of the system was illustrated by the decomposition of cocoa powder slurries in 10 % HNO_3 injected into the manifold and decomposed under stopped-flow, medium-pressure conditions.

In a series of papers [98–100], Berndt described development of a new high-temperature/high-pressure flow system for continuous decomposition of biological and environmental samples. It was shown [98] that temperatures up to 260 °C and pressures up to 300 bar can be reached in a flow system when an electrically heated Teflon-lined HPLC tube is used as the digestion capillary. Digested biological

samples (blood, liver, leaves) were collected at the outlet of the flow system. In subsequent studies [97, 98], an electrically heated Pt/Ir capillary served as a digestion tube at temperatures of 320–360 °C and pressures of about 300 bar, and could withstand concentrated acids. As a result of the totally glass-free environment, samples having high silicate content can be digested by the addition of hydrofluoric acid.

UV On-Line Decomposition

UV decomposition is a clean sample preparation method, as it does not require the use of large amounts of oxidants. Furthermore, UV decomposition is effective and can be readily incorporated into flow injection manifolds. The sample flows, in the presence of H_2O_2, H_2SO_4, or HNO_3, through a tube (PTFE, quartz) coiled around a fixed UV lamp(s). A short review of such flow systems has appeared recently [101]. Analyzers of this kind are produced by Skalar Analytical (Holland), for example.

Fernandes et al. [102] developed a manifold based on a two-stage on-line UV/thermally induced digestion procedure for oxidation purposes. The UV digestion apparatus consisted of a 4-m long PTFE tube tightly wounded directly around the UV source (15 W) to form a spiral reactor. The thermal digestion apparatus consisted of a 2-m long PTFE tube coiled in a helix and submerged in a thermostatic bath at 90 °C.

Microwave Heating (Microwave-Assisted Pressurized Flow-Through)

Many different designs for microwave-assisted flow digestion systems have been published [25, 32, 101], which open up new possibilities, primarily in fully automated sample preparation for elemental analysis.

The earliest work reported in this field was by Burguera et al. [103], who applied a flow injection system for on-line decomposition of samples and determined metals (Cu, Fe, Zn) by flame atomic absorption spectroscopy (F-AAS). The methodology involved the synchronous merging of reagent and sample, followed by decomposition of serum, blood, or plasma in a Pyrex coil located inside the microwave oven. This approach permits essentially continuous sample decomposition, drastically reduces sample processing time, and is suitable for those samples that require mild decomposition conditions (especially liquids).

According to the location of the digestion unit in the system, there are two types of manifolds described in the literature to date: before and after the injection unit. In the former arrangement, the sample is introduced into the microwave oven in a continuous flow [104] or stopped flow mode [105]; after decomposition, the injected sample flows to the microwave oven unit together with the reagent(s) to be decomposed, and is then cooled and degassed prior to its delivery to the detector

[106]. In both cases, the measurements can be performed partially or totally off-line or on-line.

Solid samples call for more sophisticated flow systems because they need to be digested in the presence of highly concentrated acids, which rapidly destroy organic matrices. A first attempt aimed at simplifying manipulation of the digest was reported in 1988 [107]. Lyophilized, finely ground and weighed samples of liver and kidney were placed in test tubes together with mineral acids, and the contents shaken before exposing them to microwave radiation to avoid violent reaction with abundant foam formation. The tubes were loaded into a covered Pyrex jar inside a domestic microwave oven operated for a specified time at a given power. Carbonell et al. [104] initiated the determination of metallic elements in solid samples using the slurry approach coupled with microwave oven digestion in a flow injection system for F-AAS determination of lead. Various natural samples (artichoke, chocolate, sewage sludge, tomato leaves), real and certified, were slurried in a mixture of HNO_3 and H_2O_2 using magnetic stirring, followed by continuous pumping around an open recirculating system, part of which (120 cm PTFE tubing) was located in a domestic oven.

A microwave-heated, flow-through digestion container (coiled Teflon tubing) was design for a commercial (Prolabo A300) focused microwave system (instead of microwave oven) and applied to the on-line preparation of biological samples, including milk, blood, and urine [108].

For extensive oxidation of organic sample constituents with nitric acid, a temperature of more than 200 °C is necessary. The PTFE tubes used, however, cannot withstand the vapor pressure of the decomposition mixture at 200 °C or more. Thus, new alternatives had to be found to overcome this limitation. One way to increase the pressure resistance of the tubes is to wrap them with plastic tape of high mechanical strength. Results from a digestion system (CEM SpectroPrep) equipped with such tubes have been published [109]. A CEM SpectroPrep system was used at moderate power to perform on-line decomposition of slurried samples of biological tissues (0.5 % mass/volume) and marine sediment (1 % m/v). The pressure thresholds of this system are near 25 bar. To achieve the desired temperature of approximately 250 °C, however, it is necessary to increase the pressure in the system up to 35 bar or so. A recently developed device enables the application of such high temperatures (250 °C) by means of a new pressure equilibrium system (with a pressure of 40 bar) [110]. The pressure equilibrium system keeps the pressure inside and outside the digestion tube (PTFE or PFA) equal, even for extremely fast oxidation reactions. The system's ability to handle only up to 1 % m/v slurries, and lower slurry concentrations for biological materials, restricts the type of sample that can be analyzed, unless the most sensitive elemental detection devices are used, such as inductively coupled plasma–mass spectrometry (ICP-MS). Therefore, Mason et al. [111] modified the SpectroPrep oven and developed a wide bore continuous flow microwave digestion system for the determination of trace metals (Cd, Cr, Mn, Ni, Pb) following aqua regia extraction. The described system demonstrated an ability to cope with real soil samples ground to a larger particle size (250 μm) and slurried without the use of surfactants.

Perhaps the current fascination for using microwave heating for on-line digestion has led to the introduction of commercial instruments based on this hybrid technique [42].

The advantages of microwave-enhanced flow systems include a significant reduction in sample preparation time, the ability to accomplish reactions that would normally be too dangerous in a closed vessel because of sudden increases in temperature and pressure, and the ability to handle transient or readily decomposed samples or intermediates. However, flow-through systems can be problematic because all samples must be homogeneous and small enough to pass through the tube, and the majority of samples require some form of processing before they can be put into the tube.

5.3.1.4 Vapor-Phase Acid Decomposition (Gas-Phase Reactions)

An alternative approach to acid digestion of the sample matrix that prevents introduction of impurities exploits gas-phase reactions. In the past four decades, several novel approaches to sample digestion procedures have been considered using inorganic acid vapor produced in one vessel to attack and dissolve material in another. A review by Matusiewicz [112] summarized analytical methods based on vapor-phase attack for dissolution and decomposition of inorganic and organic materials prior to determination of their trace element content. This approach is currently used (in open, semiclosed, and closed systems) whenever applicable because digestion using gas-phase reagents is preferable to that using reagents in solution.

The combination of hydrofluoric acid and nitric acid vapor as a digestion agent has proven effective in the preparation of samples for spectrographic determination of trace impurities in open systems. Zilbershtein et al. [113] used this approach to dissolve silicon and to concentrate impurities on a PTFE sheet. The residue and PTFE sheet were transferred to a graphite electrode that subsequently served as one electrode of the DC arc for spectrographic trace analysis.

With respect to semiclosed systems, a PTFE apparatus generating HF vapor has been specifically designed to minimize contamination during trace-element determination of ultrapure silicon, quartz, and glass [114]. The sample is placed in a PTFE beaker mounted on a perforated PTFE plate that is kept above the level of liquid HF in the chamber. Thomas and Smythe [115] described a simple all-glass apparatus for vapor-phase oxidation of up to 90 % of plant material with nitric acid. Addition of perchloric acid ensured fast and complete oxidation, and the presence of HNO_3 during the final $HClO_4$ oxidation step eliminated any danger of explosion. Klitenick et al. [116] used the same technique, with a simplified pressurized PTFE digestion vessel, for determination of zinc in brain tissue.

Some materials may not be fully dissolved by acid digestion at atmospheric pressure. A more vigorous treatment involves bomb digestion in pressure vessels designed to incorporate the techniques of a closed pressure vessel and vapor-phase digestion in a single unit (Fig. 5.9). This has the advantage of being easier to

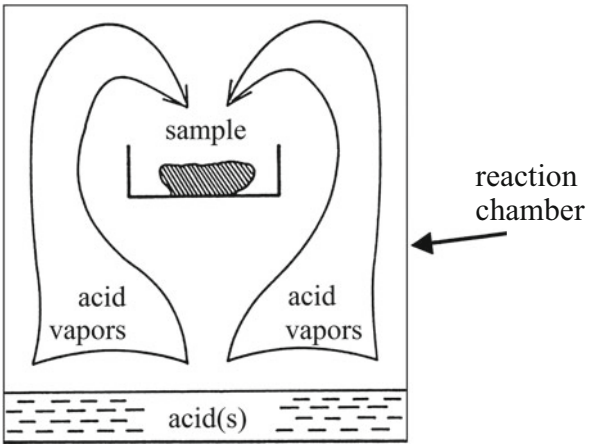

Fig. 5.9 Vapor-phase acid digestion system

construct than the apparatus described in previous papers [113–116], and it requires considerably smaller volumes of acids. Heating can be accomplished in an ordinary oven (with conductive heating) or using a microwave field.

A predecessor of this concept of closed-vessel vapor-phase sample digestion was introduced by Woolley [117]. He described low-temperature (up to 110 °C) and high-temperature (up to 250 °C) versions of the apparatus. Each device consists of an airtight PTFE vessel containing two concentric chambers: an inner chamber that holds the sample cup and an outer chamber. Both vessels were designed for the digestion of high-purity glass using relatively impure solvent acids (50:50 mixture of concentrated HNO_3 and HF). A completely closed PTFE bomb or autoclave [118] has been developed with a temperature gradient for digestion of more difficult compounds, such as siliceous material. Marinescu [119] presented an interesting development in which the conventional single-sample pressure digestion bomb was converted for multisample vapor-phase digestion. A multiplace holder for field sampling was developed to fit directly into the digestion bomb. This technique has been used for organic and inorganic solid, semisolid, and liquid samples. Kojima et al. [120] modified a sealed PTFE bomb in which the dissolution of highly pure silica with HNO_3, HCl, and HF acid vapor was possible using a PTFE vial placed in a PTFE outer vessel. A laboratory-made high-pressure digestion bomb with a PTFE microsampling device was developed by Matusiewicz [121]. This simple and inexpensive apparatus was found to be convenient for treating a small number of samples and can be easily made by modifying available PTFE bombs. It should be noted that PTFE microsampling devices can be used for both vapor-phase digestion and discrete nebulization techniques in atomic spectrometry. Vapor-phase digestion in a closed system (bomb) of high-purity materials for spectrographic determination of trace elements is a convenient and useful technique [122]. The method uses a graphite electrode with an enlarged cavity and excludes the use of a collector. A technique [123] has been developed that employs vapor-phase acid generated in the quartz vessel of a commercial high-pressure, high-temperature digestion

apparatus (High Pressure Asher HPA; Anton Paar, Austria). Small biological samples (50–165 mg) were digested in a mini-quartz sample holder (3.1 mL volume). When biological standard reference materials were digested at 230 °C and 122 bar, the residual carbon content (RCC) in the digested samples was less than 1.8 %.

Despite the success of methodologies previously proposed for closed systems with conventional heating, very few attempts to employ microwave power for vapor-phase digestions have been described. An early trial with a low-pressure microwave arrangement was unsatisfactory [124], although an interesting variant of the digestion vessel design has been proposed for dissolution and decomposition of samples [125]. The method developed was an extension of the acid vapor-phase thermal pressure decomposition of biological materials reported previously by Matusiewicz [126]. Microwave-assisted vapor-phase acid digestion, employing a special PTFE microsampling cup suitable for 250 mg subsamples, was used for digestion of marine biological and sediment reference materials with HNO_3 and HNO_3-HF, respectively, at a maximum pressure of ca. 14 bar [125]. Recently, several papers [127–131] have discussed the further application and evaluation of this pioneering concept of Matusiewicz [125], employing either commercial pressurized microwave digestion systems and quartz sample containers [127], quartz inserts [128, 129], and TFM inner vessels [128] or focused microwave ovens operating at atmospheric pressure and PTFE microsampling cups [130].

To summarize this section, use of acid vapor-phase digestion and attack of some organic and inorganic matrices is a convenient and useful method of sample preparation. Closed pressure systems are the technique of choice to avoid loss of elements by volatilization while still maintaining extremely low values for the blank (by application of isopiestic distillation of the reagents and technical grade acids).

5.3.1.5 Efficiency of Wet Decomposition Procedures

Quality control is becoming increasingly more significant in analytical chemistry. However, it is presently applied primarily to measurement techniques and not to sample preparation. For quality control in sample decomposition, it is necessary to measure and record certain parameters exactly to be able to subsequently trace the course of the decomposition process.

Complete decomposition of the sample is required to achieve reproducible and accurate elemental results by instrumental analytical methods. This is particularly the case for all voltammetric and polarographic determinations [132–135]. Interferences caused by incomplete decomposed organic compounds also occur, to a certain degree, when using atomic spectrometric methods such as AAS [136, 137], ICP-OES [138, 139], and ICP-MS [140, 141]. As noted earlier, nitric acid is the most frequently utilized sample dissolution medium. Unfortunately, the carbon contained in organic materials is only partly converted to CO_2 by HNO_3 at temperatures up to 200 °C [14]. In these cases, extending the digestion time and

increasing the quantity of nitric acid do not improve the extent of decomposition. In principle, temperature and digestion time ultimately determine the effectiveness of a digestion, with RCC serving as a useful measure of quantitative assessment [63, 142]; in other words, the highest temperatures are required to achieve a decomposition as complete as possible [143, 144]. It should be noted here that the usefulness of the decomposition technique should be judged not from a visual point of view, because a clear, colorless solution indistinguishable from water could still contain significant amounts of carbon. In closed systems, the pressure depends not only on the temperature but also on the type and quantity of the sample, the size of the vessel, and the nature and quantity of the decomposition reagent. This pressure is not responsible for the determination quality, but nevertheless it should be controlled automatically. Würfels et al. [145–148] described the extremely strong impact of residual organic compounds on elemental determinations by means of inverse voltammetry, and demonstrated that a temperature of 300–320 °C is necessary for pressurized sample decomposition with pure nitric acid to obtain a solution containing less than 0.1 % carbon. Otherwise, trace elements cannot be determined with inverse voltammetry. This was confirmed by Wasilewska et al. [149], who showed that for complete oxidation of organic compounds with nitric acid, the decomposition temperature should be raised to 300 °C. The influence of the digestion equipment (either thermal or microwave) is negligible if the digestion time employed is long enough to reach a steady-state temperature. Sample digestion with nitric acid between 220 and 250 °C leads to RCCs in the low percentage range.

The heating of digestion vessels is more and more frequently achieved by microwave technology; therefore, microwave-assisted wet decomposition is a frequently used sample preparation technique for trace element determinations in organic materials. Studies of the RCC as a measure of decomposition efficiency have been undertaken [124, 150–154]. Using gas chromatography, Stoeppler et al. [63] quantified the ashing ability of conventional pressurized decomposition. Differences between the carbon content in the original sample and the amount of carbon converted to CO_2 showed that the investigated biological and environmental samples were not completely ashed with nitric acid. Würfels and Jackwerth [155] determined the residual carbon in samples decomposed under pressure or evaporated with HNO_3. In most cases, microwave decomposition of biological material was incomplete. Subsequently, the undigested compounds were identified [145]. Parallel to the studies by Würfels and Jackwerth [155], the residual organic species in nitric acid digests of bovine liver were identified by Pratt et al. [156]. Kingston and Jassie [157] evaluated the decomposition of several biological and botanical samples that had been wet decomposed with HNO_3. Free amino acid concentrations of human urine samples were typically reduced by a factor of 10^5. This reflects the comparative efficiency of protein hydrolysis, and is not necessarily equivalent to the total carbon oxidation efficiency. Nakashima et al. [152] investigated the digestion efficiency of mixtures of HNO_3 and $HClO_4$. The total RCC in a number of decomposed marine biological reference material (NRCC TORT-1) solutions was determined and used as a relative measure of the

efficiency of various decomposition schemes. Two-stage microwave-assisted decompositions were superior to single-stage decompositions. However, even the two-stage procedures were not complete and 24 % carbon remained. The determination of residual carbon in digests of biological material with simultaneous ICP-OES analysis was described by Hee and Boyle [153] and Krushevska et al. [154]. The oxidation efficiencies of different dry and wet ashing procedures for milk samples were compared by Krushevska et al. [158], who noted that the RCCs obtained with medium-pressure microwave-assisted decompositions varied between 5 and 15 %. Oxidizing mixtures of H_2O_2 or H_2SO_4 with HNO_3 applied in a medium-pressure (11 bar) microwave system did not yield a decomposition efficiency higher than that for pure nitric acid. However, with the high pressure/temperature focused-microwave-heated TFM-Teflon bomb device, organic material was totally oxidized with nitric acid in a single-step procedure [92, 94]. Matusiewicz and Sturgeon [159] critically evaluated on-line and high-pressure/temperature closed-vessel techniques with regard to efficiency of decomposition. The completeness of destruction of biological materials (standard and certified reference materials) was characterized in terms of their RCC in the solution following digestion. Pressurized decomposition in a TFM-Teflon vessel was the most effective procedure (organic material was totally oxidized with nitric acid in a single-step procedure), whereas urine and sewage plant effluent were incompletely decomposed (between 56 and 72 %) with on-line microwave-heated decomposition using nitric acid, nitric acid and hydrogen peroxide, and peroxydisulfate oxidation. Recently, the residual weight of a bottom antireflective coating (BARC) sample was successfully used as an indicator to evaluate decomposition kinetics [160]. The weight degradation rate was independent of sample weight under various temperatures, but strongly dependent on the digestion acid volume and digestion temperature. Mathematical modeling for prediction of decomposition efficiency for the BARC sample was achieved by employing decomposition kinetics as the backbone.

Hydrogen peroxide is a very popular oxidizing reagents as it is converted to water and oxygen during the oxidation of biological material [124, 161–163]. However, experiments with mixtures of HNO_3 and H_2O_2 conducted by Matusiewicz et al. [122] showed that all versions of pressurized microwave-assisted digestion with HNO_3 and H_2O_2 gave incomplete decomposition. No significant improvement in the efficiency was achieved with 50 % H_2O_2. The extension of this observation to medium-pressure and high-temperature microwave heating provided verification of this observation [164]. Nitric acid digestion with the addition of H_2O_2 did not enhance decomposition efficiency in this study compared with use of only HNO_3. Thus, an alternative oxidizing reagent is desirable to completely and safely decompose organic carbon residues. It was found that ozone is very effective in destroying natural organic compounds [165–167], and has the potential to be used as an additional decomposition and/or finishing reagent [168].

A single digestion procedure is often insufficient for the complete decomposition of a complex matrix, leading some authors to recommend a combination of two or more techniques. Two examples suffice to illustrate the principle [95, 133]. The first example is pressure digestion followed by UV photolysis. It has been shown that

analysis of olive leaves for heavy metals by voltammetric methods leads to distorted results after "pressure digestion" alone. Reliable data can be obtained only by supplementing the digestion with UV irradiation to ensure adequate decomposition of the matrix [133]. The second example is a novel microwave-assisted high-temperature UV digestion procedure developed for the accelerated decomposition of interfering dissolved organic carbon prior to trace element determination in liquid samples. This new technique significantly improved the performance of the process of UV decomposition (oxidation) and is especially useful for ultratrace analysis because of its extremely low risk of contamination [95, 169].

In order to investigate the completeness of dissolution of inorganic materials, the recovery (or incomplete recovery) and accuracy of major, minor, and trace element determinations are usually determined. If silicates, usually the major inorganic component of many matrices (i.e., soils, sediments, sludges, ceramics, and other similar samples), are present then the use of HF to achieve complete dissolution is mandatory [170, 171].

5.3.1.6 Comparison of Wet Decomposition Techniques

A careful comparison of several digestion techniques is the only way of assuring accurate results, particularly when little experience is available with respect to the digestion of a specific matrix, or if existing reports are contradictory. The analyst must choose the sample preparation technique carefully to ensure that the system is optimal for the analyses at hand. However, there is still no universal sample preparation system. With respect to requirements specific to contamination or loss through volatilization or retention, the best choices seem to be convection-heated or microwave-assisted wet digestion, quartz-lined high-pressure wet digestion, UV digestion, and vapor-phase acid digestion. However, all of these techniques require considerable investment in apparatus. Digestion of samples in an open vessel presents a serious risk of significant analyte loss, despite the use of a reflux condenser. As far as economic aspects are concerned (low procurement, short digestion time, high sample throughput), microwave-assisted wet digestion and especially microwave-assisted pressurized on-line digestion appear to rank high. Complete degradation of many samples is achieved only through high-pressure, high-temperature Teflon- or quartz-lined pressure vessel digestion, or by combination of a closed wet digestion system with UV irradiation.

Table 5.5 summarizes the advantages and disadvantages of the wet digestion techniques discussed in the section "Wet decomposition" with respect to loss of analyte, blank levels, contamination problems, sample size, digestion time, degree of digestion, and economic aspects.

Table 5.5 Advantages and disadvantages of wet decomposition methods

Decomposition technique	Possible way of losses	Source of blank	Sample size (g)		Maximum		Decomposition time	Degree of decomposition	Economic aspects
			Organic	Inorganic	Temperature (°C)	Pressure (bar)			
Open systems									
Conventional heating	Volatilization	Acids, vessels, air	<5	<10	<400		Several hours	Incomplete	Inexpensive, needs supervision
Microwave heating	Volatilization	Acids, vessels, air	<5	<10	<400		1 h	Incomplete	Inexpensive, needs supervision
UV decomposition	None		Liquid		<90		Several hours	High	Inexpensive, needs supervision
Ultrasound-assisted decomposition	Volatilization	Acids, vessels, air					Several minutes	Incomplete	Inexpensive, needs supervision
Closed systems									
Conventional heating	Retention	Acids (low)	<0.5	<3	<320	<150	Several hours	High	Needs no supervision
Microwave heating	Retention	Acids (low)	<0.5	<3	<300	<200	<1 h	High	Expensive, needs no supervision
Flow systems									
Conventional heating	Incomplete decomposition	Acids (low)	<0.1 (slurry)	<0.1 (slurry)	<320	>300	Several minutes	High	Expensive, needs no supervision

UV on-line decomposition	Incomplete decomposition	None	Liquid		<90		Several minutes	High	Inexpensive, needs no supervision
Microwave heating	Incomplete decomposition	Acids (low)	<0.1 (slurry)	<0.3 (slurry)	<250	<40	Several minutes	High	Expensive, needs no supervision
Vapor-phase acid decomposition									
Conventional heating	None	None	<0.1	<0.1	<200	<20	<1 h	High	Needs no supervision
Microwave heating	None	None	<0.1	<0.1	<200	<20	<20 min	High	Needs no supervision

5.3.1.7 Decomposition Systems

At present, the instrumentation market offers many devices to make wet decomposition more efficient and easier to manage by means of possible automation, but this is achieved principally with microwave energy.

Wet decompositions in open vessels are undertaken with or without refluxing. Because it is crucial to adhere closely to the optimized time and temperature digestion parameters, mechanization of the digestion not only leads to higher sample throughput with less human intervention but also avoids errors. The simplest form of mechanization can be implemented through a time (programmable timer) and temperature (via an autotransformer) controlled heating block. There are many models of heating blocks on the market. A greater degree of mechanization would also incorporate control of reagent reflux during digestion.

These procedures operate batch-wise. Continuous sample handling has some advantages over discontinuous handling; the former generally better matches analytical needs. The automated wet digestion device (VAO; Anton Paar, Austria) is such a continuously operating digestion system and an ideal instrument for laboratories requiring high throughput of similar samples. It can perform all methods of wet chemical decomposition [172], allowing control of all important digestion parameters with the help of a microprocessor. Automation controls the time–temperature/pressure program for sample digestion, so that different sample materials can be processed under optimal conditions. The loading or charging of the high-pressure asher with sample material is achieved manually. A fully automated version of this high-pressure asher is not available. Berghof pressure digestion systems [173] serve for sample preparation of inorganic and organic matrices at high temperature (max. 200–250 °C) and high pressure (max. 100 and 200 bar) in pure, isostatically pressed PTFE or quartz vessels.

As noted already, three basic types of microwave-assisted digestion systems have evolved: atmospheric pressure, elevated pressure (closed vessel), and flow-through, working in two common modes: multimode cavity and focused-type (waveguide). Reviews of commercially available microwave-assisted digestion systems and vessels (summary of the vessels, ovens, and oven systems) are given in the literature [42, 174] together with specifications and features for elevated-pressure, atmospheric pressure, and flow-through units. The simplicity and efficacy of microwave digestion easily lends itself to automation and robotics. Systems have been developed that are capable of weighing samples, adding acids, capping and uncapping vessels, accomplishing microwave-assisted digestion, diluting digestates, transferring vessels, and even cleaning and reusing the vessels. Once such a system is operational, the analyst only has to supply and place the representative samples(s) in locations recognized by the system and then initiate the controlling program.

5.3.1.8 Safety of Acid Decomposition

The reagents, instruments, and operations employed in the digestion of materials are potentially hazardous, even when used as directed. The operator must always be properly protected with a laboratory coat, gloves, and safety glasses or, better still, face protection. Some concentrated fuming acids (HF, HNO_3, HCl) are to be handled only in a well-ventilated hood. Oxidizing acids (HNO_3, $HClO_4$) are more hazardous than non-oxidizing acids (HCl, H_3PO_4, HF), being more prone to explosion, especially in the presence of reducing agents such as organic matter. Perchloric acid is oxidizing only when it is concentrated and hot; it must never be brought into contact with organic matter unless diluted with nitric acid.

Acid digestion must be conducted in a fume cupboard with efficient scrubbers installed. The evaporation of perchloric acid should be performed only in an appropriate stainless steel, stoneware, or PP hood, with washing facilities to eliminate any perchlorate deposit.

Great care should be taken when using "pressure digestion" methods. Pressure digestion vessels (bombs) contain the acid fumes and are useful for rapid, one-step digestions without losses. But, again, there are restrictions; some reactions (especially spontaneous) produce potentially explosive gases that exceed the safety limits of the vessel. For instance, nitric acid and the spontaneous HNO_3 and H_2O_2 decomposition of organic matter in a closed vessel can result in explosion as a result of unintended pressure build-up within the vessel. These systems produce high-pressure spikes, which can be avoided by decreasing the sample weight or applying a gradual temperature increase.

Microwave-assisted sample digestion has its own safety requirements. As a result of the direct energy absorption and rapid heating, microwave techniques introduce unique safety considerations that are not encountered in other methods. Differences in conditions between traditional laboratory practices and microwave-implemented methods should be examined before microwave energy is used to heat reagents or samples. An excellent summary of this aspects is given in the literature [18, 19, 175].

5.3.2 Combustion

5.3.2.1 Combustion in Open Systems

Dry Ashing

The term "dry ashing" is intended to encompass all processes based gaseous or solid ashing reagents. Such a distinction relative to wet decomposition processes is not absolutely essential, but it does offer certain practical advantages. Strictly speaking, dry ashing refers to the oxidation (combustion) of a substance in air at a temperature of several hundred degrees Celsius, often in a muffle furnace or similar apparatus.

For samples that contain a lot of organic matter and are being analyzed for nonvolatile metals, dry ashing is a relatively simple method for removing organic matter. It can be used for relatively large samples (2–10 g) and requires little of the analyst's time. Classical dry ashing relies on the pyrolysis and combustion of the organic sample in a muffle furnace or laboratory flame, with the oxygen in air at 400–600 °C, to remove organic constituents. Organic matter is converted into CO_2 and H_2O [176]. The resulting inorganic "ash" residue is generally soluble in dilute acid. Crucibles used for ashing are usually made of silica, quartz, porcelain, platinum, zirconium, or Pyrex glass.

Dry ashing is rarely applied now and has largely been replaced by wet decomposition (ashing) because it has several disadvantages, such as losses caused by volatilization, very low ashing of some materials, difficult dissolution of ashed materials, and contamination. Advantages of this method are that no reagents are used and little operator attention is required.

Analytical instruments have been developed recently to dry ash samples using thermal and microwave heating; examples are the APION A dry mode mineralizer (Tessek, Czech Republic), MLS-1200 PYRO microwave ashing furnace (Milestone, USA), and MAS 7000 microwave ashing system (CEM, USA).

Low Temperature Ashing

Very gentle treatment is required for the determination of volatile elements such as Se, As, Sb, Cd, Zn, and Tl in organic materials. For this, low-temperature (200 °C) ashing with excited oxygen at a pressure of 1–5 Torr is suitable [177].

The oxygen plasma can be produced either by a radio-frequency power supply or by microwave energy (up to 300 W at 13.5 MHz). The created reactive oxygen species (free oxygen radicals and excited oxygen) react effectively with the organic sample surface (up to 2 g), forming an organic ash residue. The ash and the elements adsorbed onto the cooling finger are then solubilized by refluxing with acid (Fig. 5.10). An advantage of this method is that the elements are obtained in comparatively high concentration. This method can be used for sample preparation of all kinds of combustible solids, such as wood, paper, coal, food, or polymers.

One commercial system based on this technique is available: the Cool Plasma Asher CPA-4 (Anton Paar, Austria).

Cool Plasma Ashing (Wickbold)

The Wickbold combustion technique is suitable for processing liquid combustible samples such as petroleum products, which are difficult to decompose by other techniques [178]. In the Wickbold combustion system, an oxygen–hydrogen flame is used for sample decomposition at high temperatures (2000 °C). Liquid samples are directly introduced to the flame, whereas solid samples require a preliminary

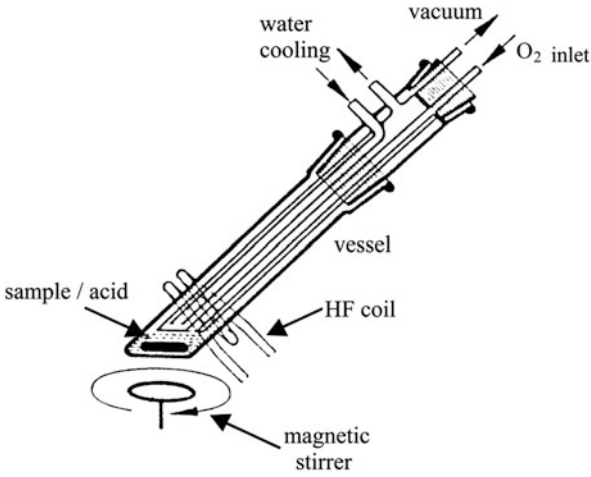

Fig. 5.10 Cool plasma asher

pyrolysis step in a pre-combustion unit. After combustion, the resulting products are condensed on a quartz surface and absorbed in a suitable solution.

A Wickbold combustion apparatus (V5; Heraeous, Germany) is commercially available.

5.3.2.2 Combustion in Closed Systems

Oxygen Flask Combustion (Schöniger)

Combustion in an oxygen flask, commonly called the Schöniger technique, offers advantages when readily volatilized elements such as halogens, Se, S, P, B, Hg, As, or Sb are to be determined. The combustion is performed with oxygen in a sealed container and the reaction products are absorbed in a suitable solvent before the reaction vessel is opened.

A simple apparatus for performing such oxidation was suggested by Schöniger [179]. It consists of a flask (500–1000 mL capacity) fitted with a ground glass stopper (Fig. 5.11). Attached to the stopper is a platinum gauze basket that holds 2–200 mg of sample. If the sample is a solid, it is wrapped in a piece of low ash-content filter paper. Liquid samples can be weighed into gelatin capsules that are also wrapped in a filter paper. A small volume of an absorbing solution is placed in the flask. During combustion, the flask is inverted to prevent escape of the volatile oxidation products. Subsequently, the vessel is opened and the resultant solution containing the analytes is removed and diluted for analysis. The time needed for decomposition using the oxygen flask is typically less than 10 min; in addition, materials and equipment are relatively inexpensive. However, the procedure needs the continuous attention of the analyst during the combustion and is commonly applied for processing only one sample at a time.

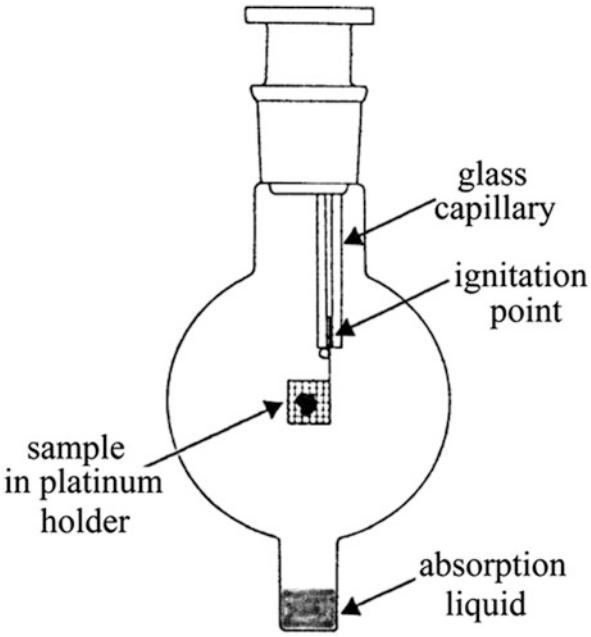

Fig. 5.11 Schöniger combustion apparatus

The flask-type combustion apparatus of the Schöniger system is commercially available (Mikro K; Heraeus, Germany).

Oxygen Bomb Combustion

The combustion bomb is the classical technique successfully applied for several matrices and analytes [180].

In this technique, the samples (as pellets) are placed in an ignition cup with two platinum wires that are connected to two electrodes (Fig. 5.12). About 10 mL of absorbing solution is added to the bottom of the vessel, which is made of stainless steel or covered with platinum. After closing, the system is pressurized with oxygen at 20–30 bar. Then, the ignition is performed using an electric current and the resulting gases absorbed after combustion. After cooling, the system is opened and the absorbing solution is removed. A relatively high sample mass (0.5 g) can be burnt in the combustion bomb with high decomposition efficiency, and the procedure takes place in less than 1 h.

Commercially available systems such as the Parr oxygen combustion bomb (Paar Instrument Company, USA) and the Bioklav pressure decomposition device (Siemens, Germany) can be employed for sample preparation applications.

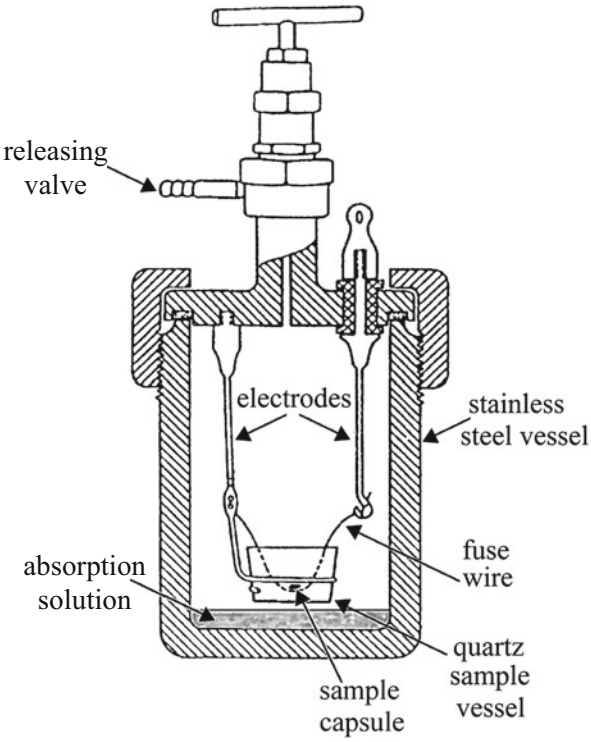

Fig. 5.12 Oxygen combustion "bomb"

Combustion in a Dynamic System (Trace-O-Mat)

The dynamic system (also called Trace-O-Mat) was developed by Knapp et al. [181] in the early 1980s and allows sample combustion with minimum contamination. The system enables not only combustion in a closed system but also further treatment. The Trace-O-Mat combustion unit VAE-II (Kürner, Germany) is shown in Fig. 5.13.

The sample is burnt in a stream of pure oxygen in a system made entirely of quartz. An essential feature is the cooling system above the combustion chamber, having a volume of only 75 mL. The cooling system is filled with liquid nitrogen and condenses all volatile traces together with the combustion products (CO_2 and H_2O). After combustion, the nitrogen is evaporated and the residual ash and the condensed volatile elements are dissolved by refluxing in 1–2 mL of high purity HCl or HNO_3 and then collected in the reagent vessel placed below. Solid samples are pressed into pellets. For liquid organic samples, a special sample holder is available. Maximum sample amounts of 0.7–0.8 g and a minimum of 1 mL of acid result in a solution with high element concentrations.

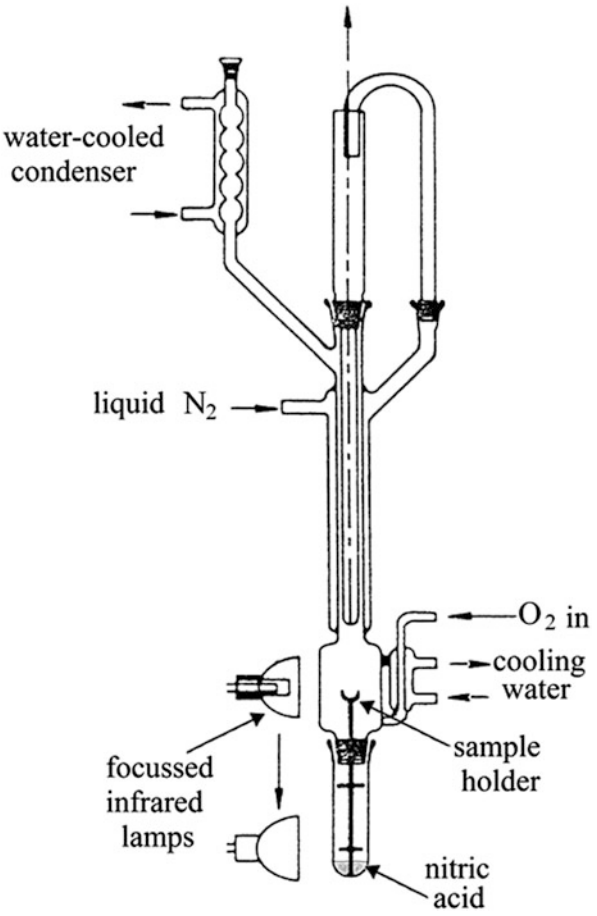

Fig. 5.13 Quartz apparatus for the combustion of organic materials in a stream of oxygen (Trace-O-Mat)

5.3.3 Fusion Decomposition

Fusion (especially alkaline fusion) is a powerful technique for both organic matrices and those with a high silica and alumina content and relatively high trace element content. In general, salt fusion is performed by mixing a sample with salts, melting the mixture with heat, cooling it, and finally, dissolving the solidified melt. The fusion-flux properties range from acidic to basic, according to the Lewis acid–base definition whereby an acid can accept and a base can donate an electron pair. The flux properties can also be termed oxidizing or reducing.

Although acid attack is a classical means of dissolving silicate samples, the use of lithium metaborate fusion is a new departure, originating in the work of Ingamells [182] who showed that a clear aqueous solution could be easily and quickly prepared from silicates. Fluxes decompose most substances at the high temperature required for their use (500–1000 °C) and the high concentration of

reagent brought into contact with the sample. The sample in the form of a very fine powder is mixed with a tenfold excess of the flux in a graphite or platinum (sometimes nickel or zirconium) crucible. The crucible is then placed in a muffle furnace at 500–1000 °C for a few minutes to several hours to give a "melt." After cooling the melt is dissolved.

Different fluxes can be used. Basic fluxes employed for the attack of acidic materials include carbonates, hydroxides, peroxides, and borates. Oyrosulfates can be used as acidic flux. If an oxidizing flux is required, sodium peroxide can be used. As an alternative, small quantities of alkali nitrates or chlorates can be mixed with sodium carbonate. Basic and acidic fluxes are dissolved in an acid or basic medium, respectively.

The addition of fluxes increases the risk of raising the blank value, as a result of the amount of flux required for successful fusion. In addition, the final aqueous solution obtained from the fusion has a high salt concentration, which can cause difficulties in subsequent steps of the analysis. The high temperatures required for a fusion increase the danger of volatilization losses.

These disadvantages make fusion a less than ideal technique for extreme trace element determination. However, for the determination of major, minor, and even some trace elements in such matrices as fly ash, silicates, slags, and dust good results can be obtained.

5.4 Conclusions and Future Trends

The chief methods used for the decomposition of organic and inorganic samples have been evaluated. A brief summary of applications of these techniques to various sample matrices is presented in Table 5.6. The variety of approaches currently available for the decomposition of solid and liquid samples allows the most suitable method to be selected for each application, depending on both the matrix and type of analyte, and subsequent steps to be developed in order to complete the analytical process. In spite of this, sample decomposition should not be looked at as an isolated step, but one that needs to be integrated into the entire analytical process.

Attention has been focused on decomposition at elevated temperature and pressure. High-pressure decomposition with its large decomposition temperature range is the most universal decomposition system at present. It is the technique of choice for the vast majority of both inorganic and organic materials. New ways to further increase the efficiency of sample preparation should appear with development of hyphenated decomposition techniques. A novel, microwave-assisted, high-temperature UV digestion for accelerated decomposition of dissolved organic compounds or slurries has been developed [95]. This new technique is ideal for extreme trace analysis because of the low blank values and low acid concentration. In addition, this digestion method can be used for the determination of non-metals by ion chromatography. Alternatively, within the limits of Teflon-lined digestion

Table 5.6 Summary of application of total wet decomposition procedures to the analysis of materials (determination of elements)

Material/matrix/sample	Required acid(s)[a]	Decomposition technique (mode)[b]
Water(s)	H_2O_2, HNO_3	UV irradiation
Environmental samples		
Coal	HNO_3, HCl, HF	Open or closed system
Coal fly ash	Aqua regia[c]+HF[d]	Open or closed system
Dust	Aqua regia+HF	Open or closed system
Catalysts	Aqua regia	Open systems
Waste materials		
Sewage sludge	HNO_3, HCl	Open or closed or flow systems
Waste water	HNO_3	Flow systems
Botanical systems		
Botanicals	HNO_3+H_2O_2+HF	Open or closed system
Plants	HNO_3+H_2O_2+HF	Open or closed system
Clinical	HNO_3	Open or closed system
Marine	HNO_3	Open or closed system
Forensic	HNO_3	Open or closed system
Food(s)	HNO_3	Open or closed system
Beverages	HNO_3, H_2O_2	Open or closed or flow systems
Silicates		
Soils	Aqua regia+HF	Open and/or closed systems
Sediments	Aqua regia+HF	Open and/or closed systems
Glasses	HF	Open systems
Geological samples		
Rocks	Aqua regia+HF[e]	Open or closed systems
Ores	Aqua regia+HF	Open or closed systems
Minerals	HF+H_2SO_4, HCl	Open systems
Petroleum products		
Fuels	HNO_3+HCl	Open or closed systems
Oils	HNO_3+HCl	Open or closed systems
Drugs and pharmaceuticals	HCl, HNO_3	Open systems
Metals		
Ferrous	HNO_3+(HF or HNO_3 or	Open systems
Non-ferrous	H_2SO_4)	Open systems
Alloys	HCl or HNO_3 or HF	Open systems
Steels	Aqua regia+HF	Open systems
	HCl+HNO_3, $HClO_4$[f]	
Chemicals	HCl, HNO_3, HF, H_2SO_4	Open or closed systems
Polymers	HCl, HNO_3, HF, H_2SO_4	Open or closed systems

(continued)

Table 5.6 (continued)

Material/matrix/sample	Required acid(s)[a]	Decomposition technique (mode)[b]
Refractory compounds[g]		
Ceramics	HNO_3, HCl, HF, H_2SO_4, H_2O_2	Open or closed systems
Composites	HNO_3, HCl, HF, H_2SO_4, H_2O_2	Open or closed systems
Nuclear materials	HNO_3 or HCl, H_3PO_4, $HClO_4$	Open or closed systems

[a]Concentrated acids are usually employed; H_2O_2 is 30 %. In most cases alternative decompositions are possible depending on the requirements of the analyst
[b]Conventional or microwave
[c]Unstable
[d]Use only Teflon vessels; the addition of HF is required to obtain quantitative recoveries for Cr
[e]Addition of H_3BO_3 to neutralize HF by forming tetrafluoro-boric acid
[f]Danger of explosion
[g]Certain refractory materials are not decomposed; these must be solubilized by fusion

vessels, improvement in decomposition efficiency can be achieved by adding optimum concentrations of strong oxidizing agents, such as ozone or oxygen, which appear to be efficient decomposition agents for the treatment of biological material. Again, this has the advantage that the agent does not contribute to the analysis blank. It should be mentioned that vapor-phase acid digestion offers an alternative solution to these problems; it allows reduced concentration of acid in the digestate and the possibility of using a technical grade acid without any deterioration of the analytical blank. Another example where significant improvement in decomposition and dissolution was obtained is the use of a reactor that combines microwave and ultrasound energy [42]. It is expected that these two methods could open a new research field, "combined digestion techniques."

It can be said with certainty that most digestions will be performed in the future by means of microwave assistance. Progress has been made over the past few years in reducing systematic errors and improving detection limits with microwave digestion, as well as its automation. A noticeable trend toward pressurized closed-vessel systems permitting high-temperature decomposition compatible with trace analysis has occurred. Some researchers advocate high-pressure (100 bar) digestion at 250–300 °C to destroy interferences in refractory compounds and manufacturers are working to devise sample vessels that can withstand these conditions.

There has been a growing trend in recent years toward development of fully automated on-line analysis techniques. Microwave-assisted high-pressure flow digestion systems with PTFE or PFA tubes for digestion temperatures up to 250 °C open up new possibilities for fully automated sample preparation [42]. On the other hand, there has been development of new high-temperature/high-pressure flow digestion systems that incorporate resistively heated capillaries for the continuous digestion of various samples, coupled with atomic spectrometric instruments [98–100]. It is predicted that flow systems will become dominant for liquid samples and slurries and extend the analytical capabilities of instrumental methods

by combining sample preparation with simultaneous analysis, using only micrograms of sample and microliters of reagents. The final goal of these studies should be the adaptation of standard batch digestion methods to on-line systems combining flow-through digestion directly to analyzers.

It is evident that wet decomposition methods remain a fertile area for development. New digestion techniques need to be designed that address the limitations of the instrumentation and maximize its potential. Development trends for conventional and microwave instruments will focus on sample throughput, enhanced vessel performance specifications, the use of new materials, further refinement of in situ vessel control (direct temperature and pressure, incident and reflected microwave power), and computer-controlled sample digesters with automated capability.

Finally, the development of automated methods for wet decomposition of solid samples without human participation can only be achieved with the use of a robotic station [183]. Nevertheless, a number of auxiliary energies and commercially available modules can facilitate and/or accelerate this time-consuming step of the analytical process (i.e., obtain the analyte(s) from a solid sample in the form of a solution).

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Chapter 6
Extraction Methods in Trace Analysis

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6.1 Introduction

Extraction processes and phenomena commonly occurring and easily observed in nature and the environment are of extreme importance and of enormous potential. Therefore, extraction processes have been widely applied in many areas of daily life, with special utilization in chemical and related technologies as well as chemical analysis.

Extraction techniques are universal, standardized, and commonly applied sample preparation methods (see for example [1–4]). Development of extraction methodology in the past 20 years has been eventful and impressive. Universalism of these techniques is associated with applicability to analyses of a great diversity of organic compounds (including DNA and RNA) and elements and their speciation forms, which are present in different sample matrices both in trace and macro quantities.

Extraction can be used as an efficient and selective sample preparation method before analysis by chromatographic, spectroscopic, electroanalytical, or electrophoretic methods (see for example [5–10]). International norms from the International Standards Organization, US Food and Drug Administration, and US Environmental Protection Agency recommend application of extraction methods in analysis of food products and environmental and pharmaceutical samples. Novel ideas and new views concerning extraction have led to many controversies about terminology and to reallocation and softening of the boundaries between extraction and other analytical sample treatment techniques.

Extraction is a mass exchange process in a multiphase and multicomponent system that involves transfer and distribution of one or more components into two

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immiscible phases; therefore, thermodynamic and kinetic considerations, or at least one of these, must be taken into account. Extraction processes can be described by appropriate thermodynamic and kinetic equations as well as by thermodynamic functions. In some cases, for instance, liquid–liquid extraction, the equilibrium process is considered; in other circumstances, such as leaching of solid samples or solid-phase extraction (SPE), the process is treated as unbalanced.

In extraction of organic compounds, derivatization reactions are often recommend because they both ensure high efficiency of isolation of some organic species and result in a more convenient form (by changing a selected functional group) for detection and sensitivity during instrumental measurement. Some newly developed and more sophisticated extraction methods fulfill the requirements of green chemistry and considerably reduce consumption of energy and chemicals, as well as allow specific recycling of the extraction systems used.

It is only possible to present some selected problems and techniques relating to extraction phenomena in this monograph devoted to trace analysis; of course, every choice, as well as the criteria used for selection, is subjective.

6.2 Classification of Extraction Methods and Analytical Performance

Numerous extraction methods and techniques have been developed and reported, especially if one takes into account the variety of modifications. The most common and simple general classification of these methods is similar to that introduced in chromatography and based on the kind of phase to which the analyte is transferred. One can distinguish the extractions as liquid, solid, gas, and supercritical fluid phase extractions. More precise description specifies the two phases between which the analyte is distributed (e.g., liquid–liquid or solid–liquid [leaching] extractions). The latter methods are all called solvent extraction.

There are number of methods and techniques used in extraction procedures:

- Periodical and continuous
- With and without any additional energy supplied to the system (generally, microwave or ultrasonic energy)
- Single or multiple
- Counter-current and parallel current
- In open and closed systems (low-, atmospheric-, or high-pressure devices)
- Column extraction
- With and without accelerated solvent extraction

The extraction methods can be also categorized according to the mechanisms of phenomena involved in the separation processes. Some authors take into account the type of analyte and/or sample; others consider applicability for chromatography and instrumental aspects. Various new extraction methods reported in the past two

decades can be quite easily categorized according to the classification outlined above. For example, hollow-fiber liquid-phase microextraction, accelerated solvent extraction, supramolecular solvent-based extraction, and dispersive liquid–liquid microextraction fall within the solvent extraction group. It seems reasonable to distinguish two categories of microextraction, the first based on SPE (e.g., sorbent-based microextraction, stir-bar sorptive extraction) and the other based on solvent extraction.

Ensuring high-quality analytical performance in trace analysis, if separation of sample components by extraction is indispensable, requires implementation of the appropriate extraction method and establishment of suitable operational parameters to ensure a high efficiency of extraction. Selection of extraction conditions is crucial for quantitative recovery of analyte, or at least for sufficient effectiveness. If an aqueous solution is one of the extraction phases, problems such as complexation, hydrolysis, and solvation can play an important role. Extraction of elements from aqueous to organic phase often requires selection of appropriate ligands and control of pH.

In the case of solvent extraction, an eluent (solvent) should, among others, have the following characteristics:

- Very good solubility of the analyte
- Acceptor and donor phase immiscibility
- Nontoxicity
- Nonflammability
- Availability and low pricing
- Stability and inactivity
- Boiling temperature considerably different from that of the primary solvent

The method of trace measurements used must be also taken into account. For example, volatile solvents are required for gas chromatography coupled with mass spectrometry (GC-MS) whereas nonvolatile solvents are used for high performance liquid chromatography (HPLC). Low surface tension can lead to creation of an emulsion and result in a longer time for phase separation.

SPE consists of analyte immobilization on the sorbent in the first stage, followed by selective elution of the components of interest by an appropriate solvent. Solid, adsorptive phases are selected according to their high affinity for the analytes (significantly higher than between analyte and donor phase). Proper eluent choice is the second factor influencing SPE efficiency.

6.3 Selected Extraction Methods

During selection of the extraction methods to include in this chapter we considered the popularity of the method, maturity, simplicity of application in laboratory routine, cost, and prospects for development by further modification. New promising extraction modifications can be found in numerous current review papers. We

have also omitted some other methods (such as the Soxhlet method) that are presented in the application section of this book.

6.3.1 Solid-Phase Extraction

The term "solid-phase extraction" was introduced by personnel of the J. T. Baker Company in 1982. The method consists of retention of the analytes from a liquid or gaseous sample to a solid stationary phase and subsequent removal of analytes using an appropriate eluent. The main purpose of SPE is isolation and preconcentration of compounds of interest or sample clean-up and simplification of the matrix. Application of this sample preparation technique also allows extract fractionation. As a result of significant reduction in the volume of organic solvents used, high recovery, and the possibility of process automation, SPE is a good alternative for conventional liquid–liquid extraction. According to their affinity for the compound of interest, stationary phases are classified as follows:

- *Nonpolar*: for nonpolar and or moderately polar compounds in a polar sample matrix
- *Polar*: for extraction of polar or moderately polar analytes from nonpolar solutions
- *Ionic*: for isolation of anionic or cationic compounds

Many novel sorbents have been invented for simplification and improvement of extraction procedures [11–13].

Silica gel modified with carbon chains, including the most popular, $-C_{18}$ (octadecylsilica), in which alkyl groups have 18 atoms of carbon, are usually applied in reversed phase separations. Different alky or aryl groups are used for modification of silica, such as $-C_2$, $-C_4$, $-C_8$, –CN, and $-NH_2$. All these functional groups are hydrophobic; therefore, retention of analytes is a result of nonpolar–nonpolar attractive forces or dispersion forces. Sorbents of silica gel modified with carbon chains are stable over a narrow pH range.

Polymer sorbents (such as styrene–divinylbenzene copolymer) or graphitized carbon black, which retain analytes as a reversed phase are used the most frequently. These sorbents are stable at a wide range of pH and have well-expanded surfaces; therefore, most of the analytes dissolved in water are well retained. Retention of analytes on this kind of sorbent is based mainly on the size and shape of the analyte. Recently, chemically modified polymer sorbents with functional groups such as acetyl, hydroxymethyl, benzoyl, or carboxybenzyl have become available on the market. Chemical introduction of different functional groups into a polymeric resin improves the efficiency of SPE by providing better surface contact with aqueous samples. Chemically modified polymer sorbents are highly hydrophobic and recovery obtained for them is better than with unmodified analogs. Polymeric gels with well-defined pore size allow separation of mixture constituents on the basis of molecule size, and such a process is called gel filtration.

For example, Sephadex adsorbent is used for separation of low and high molecular weight molecules.

Adsorption of analytes on ion-exchange sorbents is based on electrostatic interactions between charged functional group in the compounds of interest and the positively or negatively charged groups on the stationary phase (bonded to the silica surface).

To improve sorbent selectivity, and thereby to increase separation process efficiency, multifunctional sorbents have been developed. These kinds of sorbents have two functional groups that can offer mixed retention mechanisms (i.e., hydrophobic, ionic and/or pi-cation interactions). Different interaction mechanisms give efficient retention of compounds with different polarities and acidities. Such sorbents are applied in analysis of some pharmaceuticals, food samples, biological fluids, animal tissues, and wastes.

The most specific and selective sorbents are the new stationary phase types, immunosorbents and molecularly imprinted polymers (MIPs). Immunosorbents are sorbents with a high degree of molecular selectivity because of a specific antibody attached to the silica surface for the purpose of removing the corresponding antigen from solution [14]. Selectivity enhancement is caused by specific antigen–antibody interactions (immunoaffinity). MIPs are three-dimensional polymer networks obtained as a complex between a template of target analyte and a functional monomer [15]. After removing the template from the polymer, specific recognition cavities are formed that are complementary in shape, size, and chemical functionality to the template molecule. Most often, analyte binding is provided by intermolecular interactions such as hydrogen bonds, dipole–dipole interactions, and ionic interactions between the template molecule and functional groups of the polymer matrix.

Usually, sorbents are placed in the extraction columns or tubes; however, many sizes and types of stationary phase extraction cartridges or extraction disks are now available commercially.

Separation of analyte from the stationary phase usually takes place with the aid of a selected solvent (eluent), sometimes by desorption. Sequential elution, carried out with the aid of two or more elution solvents is employed for selective separation of two or more compounds of interest or groups of similar analytes. Several factors, such as kind of analyte of interest, matrix composition, type of stationary phase, and detection method, should be considered during selection of eluting solvents. For polar stationary phases, the elution strength is determined by solvent polarity and polarizability. In the case of nonpolar sorbents, the elution strength depends on nonspecific van der Waals forces. For selection of eluent, other criteria such as solvent viscosity, solubility in water, refractive index, UV cutoff wavelength, suitability for a specific detector, and environmental comparability must be taken into consideration. An eluotropic series records solvents by their relative ability to remove an analyte from a given adsorbent. Examples of application of SPE in trace analysis of pharmaceutical products and biological and environmental samples are given in Table 6.1 [16].

Table 6.1 Examples of application of solvent phase extraction methods for extraction of pharmaceutical traces

Analyte	Sample	Sorbent type	Elution solvent	Determination method
β-Lactam antibiotics (e.g., penicillin G, ampicillin, amoxicillin)	Wastewater	Oasis Max (polymer)	Tetra-*n*-butylammonium, hydrogen sulfate in methanol	HPLC-UV
Tetracycline, sulfonamides, macrolides	River water	Oasis HLB (polymer)	Methanol	LC-MS
Sulfonamides, tetracycline	Milk	Oasis HLB (polymer)	Methanol	LC-ESI-MS
Tetracycline, macrolides, sulfonamides	Soil, liquid sample after PLE	Oasis HLB (polymer)	Methanol	LC-ESI-MS
Tetracycline, macrolides, sulfonamides	Sediment, liquid sample after LLE	Oasis HLB (polymer)	Methanol	LC-MS
Tetracycline	Tissue, liquid sample after LLE	MAA as functional monomer, EGDMA ascrosslinker	KOH in methanol	HPLC-UV

PLE pressurized liquid extraction, *LLE* liquid–liquid extraction, *MAA* methacrylic acid, *EGDMA* ethylene glycol dimethacrylate, *HPLC* high-performance liquid chromatography, *LC* liquid chromatography, *ESI* electrospray ionization, *MS* mass spectrometry

The types of organic species extracted using SPE and the variety of samples analyzed has increased impressively and is mainly a result of the application of new smart materials as sorbent materials (e.g., immunosorbents, molecular printed polymers, carbon nanomaterials).

Only basic information has been included in this section on this very important technique. More details concerning a great variety of specially designed modern materials used as solid sorbents and their future prospects are presented in review papers [17–19].

6.3.1.1 Solid-Phase Microextraction

Solid-phase microextraction (SPME) is a fast and solventless modification of SPE techniques [20]. SPME involves the use of fiber (fused silica fiber or polymer-coated fused fiber) coated by an adsorbent. The method is applied for extraction of different kinds of both volatile and nonvolatile analytes from different kinds of media, which can be in liquid or gas phase. In the direct extraction mode, coated fiber is immersed in the sample for a defined time, until equilibrium between the sample matrix and the solid phase is reached. The analyte is transported by diffusion directly to the extracting phase. In the next stage, the compound of interest

is desorbed and determined. In the case of volatile compounds, determination desorption occurs at high temperatures directly in the gas chromatograph injection system or in a carrier gas stream introduced into the chromatograph [21, 22].

At present, polydimethylsiloxane, polyacrylate, and carbowax™-divinylbenzene or polydimethylsiloxane-divinylbenzene are most often used as stationary phases. Nonpolar analytes are isolated with the aid of polydimethylsiloxane; for separation of polar compounds, polyacrylate is applicable.

Development of SPME techniques must meet the requirements of green chemistry principles for implementation in industry, laboratories, and education [23]. Modifications of the SPME technique allow significant reduction in solvent use, minimization of sample volume needed for analysis, and direct analyte determination. Examples of miniaturized techniques are stir bar sorptive extraction (SBSE) [24–26] and microextraction by packed sorbents (MEPS) [27–29]. In SBSE, analytes are extracted into a polymer coating on a magnetic stirring rod. Most SBSE applications involve the use of thermodesorption. MEPS is usually an automated technique in which polysiloxane-based sorbent is packed into a cartridge that is integrated into a microliter syringe. Minimization of sample volume for analysis, reduction in solvent volume for elution, and reduction in time needed for sample preparation and injection are the main advantages of this method. Examples of MEPS applications in trace analysis of environmental, food, and biomedical samples are presented in Table 6.2 [30, 31].

6.3.1.2 Headspace Techniques

Term "headspace" means the gas layer above a gas, liquid, or solid sample [32]. Headspace extraction consists in partition of volatile or semivolatile organic constituents from the sample matrix into the headspace gas volume. During this

Table 6.2 Application of microextraction by packed sorbents in trace analysis

Analyte	Sample	Sorbent	Determination method
Amphetamine	Urine, hair	Polydimethylsiloxane fiber	HPLC
Lidocaine	Blood serum, urine	Polydimethylsiloxane fiber	GC-FID, HPLC-UV
Heterocyclic aromatic amines	Food	Carbowax, carbowax-divinylbenzene, polydimethylsiloxane-divinylbenzene, polyacrylate	HPLC-UV
Pesticides/ organophosphorus	Honey	Polydimethylsiloxane	LC-MS
Cr (III)	Water	Polymer-coated silica fiber	GC-FPD

GC-FID gas chromatography with flame ionization detector, *HPLC-UV* high performance liquid chromatography with UV detector, *LC-MS* liquid chromatography coupled with mass spectrometry, *GC-FPD* gas chromatography with flame photometric detector

kind of extraction, volatile sample constituents diffuse from the matrix into the gas phase, thereby forming headspace gas. The classical static headspace technique (vapor-phase extraction) includes thermal evaporation of the compound of interest from sample sealed in a vial. Diffusion equilibrium declines at a defined temperature, at which the headspace vapor is sampled. Then, the atmosphere above the sample is acquired and introduced into the injection port of the GC apparatus. A more effective version of static headspace extraction is multiple headspace extraction, in which repeated consecutive extractions of the same sample headspace gasses are performed for the same sample vial, which allows measurement of the total amount of analyte present in the sample.

In dynamic headspace extraction (purge and trap extraction mode), the constant passage of carrier gas through a warmed sample (purge), followed by trapping of the purged volatiles on a sorbent (trap) and desorption into a gas chromatograph take place.

Headspace solid-phase microextraction (HS-SPME) is a rapid and solvent-free modification of the SPME technique in which a fine fused silica fiber with a polymeric coating is inserted into a headspace gas to extract organic compounds and directly transfer them into the injector of a gas chromatograph for thermal desorption and analysis. In this technique, the quantity of compounds extracted onto the fiber depends on the polarity and thickness of the stationary phase as well as on extraction time and concentration of volatiles in the sample.

Examples of the application of headspace extraction are flavors in food products, volatile organic compounds in soils, and residual solvents in pharmaceutical products [33, 34]. The main advantages of headspace extraction are minimal sample preparation and the possibility for direct introduction of headspace gas into the gas chromatograph.

Only basic information has been included in this section. More details on the fundamentals, theory, transfer of gas to GC, applications, and devices can be found in a number of guides and papers [35–37].

6.3.2 Membrane-Based Extraction

Membrane-based extraction, conceived in the 1960s, is widely used because of its selectivity, high enrichment factor, and possibilities for automation. The membrane is a selective barrier that solute molecules pass through from the sample (donor phase) to the receiving side as a result of a concentration gradient. In some cases, the compound of interest is removed from the receiving phase by a gaseous, liquid, or solid phase. Extraction of volatile compounds from gaseous sample to gaseous acceptor through a membrane is called permeation.

The transport of molecules is faster if the membrane area is larger and membrane thickness smaller. Also, the temperature (the diffusion coefficient rises with increasing temperature) and solute partitioning coefficient influence diffusion.

The efficiency of membrane-based extraction is related to the diffusion and partitioning coefficients [38–40].

Membrane-based extraction techniques can be classified on the basis of membrane structure (porous and nonporous) or on the number of the phases taking part in the extraction process [41, 42]. Filtration, dialysis, and electrodialysis are separation techniques involving the presence of a porous membrane. In dialysis, both donor and acceptor solution are in continuous contact through the membrane pores. The pore dimensions allow diffusion of small molecules. Diffusion is the result of a concentration gradient between aqueous donor and receiving sides of the membrane. To increase process efficiency, disposal of the receiving phase is carried out. Sometimes dialysis leads to significant purification of the solution from macromolecules such as proteins and humus compounds. Preconcentration of the analyte is usually not possible. For that purpose, coupling of dialysis and SPE is necessary.

There are also techniques involving the use of nonporous, solid or liquid membranes that separate the donor phase from the receiving phase by an evident phase boundary. Most often used are three-phase systems (donor phase, membrane, and acceptor phase) or two-phase systems, in which one of the surrounding phases is the same as the membrane. Solid membranes are made of chemically resistant, hydrophobic polymers (PTFE, PVDF, PS, PP, silicates), metals (Pd alloys), or ceramic materials. Channels of membrane modules have a volume ranging from 10 to 1000 μL and, according to their geometry, can be classified as planar or fibrous. For setting up a membrane system, two modes can be used: the membrane can be immersed in a sample (membrane in sample, MIS) or the sample can be introduced into a membrane (sample in membrane, SIM). In both systems, only a small amount of sample is in direct contact with membrane, because ratio of the membrane surface area to the sample volume is small.

Liquid membranes exist in the form of a wide layer, emulsion, stationary phase, hybrid, or polymer. The simplest, but only intermittently applied in analytics, is a wide layer membrane system in which the organic phase separating two aqueous phases is from a few to a dozen centimeters thick and often contains a substance supporting analyte transport. Liquid emulsion membranes (LEMs) are formed as a result of emulsification of two immiscible phases, with droplets dispersed in the third phase. The main advantages of emulsion membranes are a large area of membrane phase and small acceptor phase volume, which results in high extraction efficiency and enrichment of analyte concentration. After analyte isolation and separation (usually gravitational) of the emulsion from a donor phase, the emulsion is broken using electrical or (less often) chemical or thermal means. An important group of liquid membranes that are widely applied in analytical laboratories are supported liquid membranes (SLMs). In this type of membrane, organic solvent is held by capillary forces in pores of a flat porous membrane support sheet or in porous hollow fibers. Foil thickness (and membrane thickness) is 100–200 μm and the volume of immobilized organic solvent is of the order of microliters. As organic phase, aliphatic alcohols, hydrocarbons, or ethers can be used (e.g., dihexyl ether, dioctyl phosphate, or kerosene). The driving force of the SLM extraction process

could be, for example, the pH, concentration, or potential difference between donor and acceptor phases. Solute transported from the donor phase dissolves in the organic membrane, and is subsequently transferred into the second aqueous phase as a result of a concentration gradient between donor and acceptor phases. Extraction and re-extraction processes depend on the analyte separation coefficient between aqueous phase and membrane. Diffusion flux can be maintained if the substance of interest is chemically converted into a form insoluble in the organic membrane. Selective transport is possible and could be facilitated by the presence in the liquid membrane of compounds that can form complexes with the analyte. SLM extraction is mainly applied for extraction of polar components, such as organic and inorganic acids and bases or metal ions. More stable than SLMs are polymer inclusive membranes (PIMs) formed by instillation of polymer solution containing plasticizer and an appropriate analyte carrier. In this type of membrane, the organic phase fills the whole volume of pores in the polymer foil. Most often, silicon rubber, polyvinyl chloride, or cellulose triacetate are used. Analyte transport is provided by a carrier, a complexing agent, or ion exchanger [41].

Application of polymer membranes to separation of aqueous and organic phases in liquid–liquid extraction processes is called microporous membrane liquid–liquid extraction (MMLLE). An organic acceptor solvent, filling the pores of the hydrophobic membrane, stays in direct contact with the aqueous phase near the membrane surface, where mass transfer takes place. This kind of extraction is similar to SLME, but takes place in a two-phase system and is slower and less selective because of the absence of carrier agent. Because the polymer membranes are insoluble, an arbitrary combination of aqueous and organic phase is possible and the extraction efficiency mainly depends on the partition coefficient.

A significant advantage of membrane-base extraction is the possibility of automation by interfacing to analytical instrumentation and processing in a flow system [42]. Mass spectrometers, chromatographs and, in the case of analysis of metals, atomic spectrometers are most often used as detectors. In combination with mass spectrometry, the membrane can be placed inside the vacuum system and the analyte directly introduced into the ionization source. Membranes used for permeation of gases or volatile compounds are typically hydrophobic nonporous silicon membranes; therefore, coupling of membrane extraction and gas chromatography is more complex. Table 6.3 lists some selected recent applications of membrane-based extraction in trace analysis [38–42].

6.3.3 Microwave-Assisted Extraction

During microwave-assisted extraction (MAE), microwave energy is applied to heat a solvent or/and a sample in order to speed up transfer of the compound of interest from the sample to the solvent [43, 44]. Microwaves are an electromagnetic radiation with wavelengths ranging from as long as 1 m to as short as 1 mm, with frequencies between 1 and 100 GHz.

Table 6.3 Selected applications for membrane extraction in trace analysis

Analyte	Sample/matrix	Membrane extraction technique
Medicaments	Aqueous solutions, biological fluids	SLM, MMLLE
Carboxyl acids	Waters, air, soil	SLM
Herbicides, fungicides	Natural waters, food	MMLLE, PME
Amino acids	Synthetic aqueous solutions	ELM, SLM
Metals and their speciation forms	Waters, urine, waste, brine	SLM, ELM, PIM
Iodine; I_2	Aqueous solutions	PIM
Phenols	Natural waters, fuels	SLM, PME, MMLLE

SLM supported liquid membrane, *MMLLE* microporous membrane liquid–liquid extraction, *ELM* emulsion liquid membrane, *PIM* polymer inclusion membrane

Table 6.4 Physical constants for selected solvents

Solvent	Dielectric constant	Dipole moment (20 °C)	Boiling point (°C) ($p = 101.3$ kPa)
Acetone	20.7	2.69	56.3
Acetonitrile	35.9	3.44	81.6
Ethanol	24.6	1.69	78.3
Hexane	1.89	0.08	68.7
Methanol	32.7	2.87	64.6
2-Propanol	19.9	1.66	82.3
Water	78.9	1.87	100

Microwave radiation can be transferred by different compounds by dipole polarization or ionic conduction. Dipole polarization determines heating of polar compounds consisting of molecular dipoles (e.g., water, acetic acid, chloroform). In this case, microwave energy stimulates dipole rotation and reorganization with the applied field. In ionic compounds, microwave energy causes ion movement with the field. This migration of ions and resistance of the solution to this flow result in heating of the solution. In a paper from 1986, Ganzler et al. [45] for the first time presented the extraction of crude fat and antinutrients from food, and pesticides from soil. Application of microwave energy for liquid–liquid extraction leads to significant reduction in extraction time. Unlike conventional heating, microwaves are absorbed by the whole sample without affecting the vessel material, because microwave energy is transferred to materials through molecular interactions and the electromagnetic energy is converted into thermal energy. Dipole molecules and ions absorb microwave energy, but nonpolar solvents such as hexane do not get warm in response to microwave action. The greater the solvent dielectric constant, the more thermal energy is released and the faster the extraction solvent or sample matrix reach the required temperature. Table 6.4 shows selected physical parameters of solvents used as extractants in MAE.

Three basic mechanisms of MAE can be distinguished:

1. Sample is extracted by one solvent or a mixture of solutions that absorb microwave energy
2. Extractant is a mixture of high and low dielectric constant solvents
3. High dielectric constant sample is leached with an agent that does not absorb microwave energy

Generally, two set-ups for conducting MAE are applied in laboratories: closed vessels systems allowing for temperature and pressure adjustment and control, and open vessel systems for procedures carried out under atmospheric pressure. In the open vessel systems, maximal temperature is determined by the temperature at which the extractant boils. In those systems, absorption of microwave radiation occurs in the whole sample; therefore, heating is effective and homogeneous. The main disadvantage of open vessel systems is the possibility of volatile compound loss. This can be reduced by application of a reflux system fitted into the top of the extraction vessel.

Optimization of the MAE procedure consists in choosing an appropriate solvent (or solvent mixture) and others factors such as solvent-to-feed ratio, extraction time and temperature, microwave power, and matrix characteristics (including water content).

In most reported procedures, the extractant has a high dielectric constant and strongly absorbs microwave energy (mechanism 1). Examples of single extractant application are extraction of taxanes with methanol or ethanol or the use of tetrahydrofuran for leaching of low molecular weight oligomers. Mixture of two polar solvents, acetonitrile and methanol, was employed for felodipine extraction from pharmaceuticals. For leaching of atrazine traces and its derivatives from soil, dichloromethane and methanol are utilized [46].

The mixture of polar and nonpolar solvents often employed for extraction of polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs) is a hexane and acetone combination. Ethyl acetate and cyclohexane are used for extraction of chlorinated organic compounds [46].

For high dielectric constant samples the use of solvents that do not absorb microwave energy is most effective. Extraction of thermally unstable analytes from high humidity samples is an example of such extraction [46, 47]. Microwaves heat the moisture in the matrix, evaporating it and generating internal pressure in the cell. This causes the cells to break and release the solutes, consequently improving extraction efficiency. This process differs from classical solvent extraction, in which solvent diffuses into the matrix and releases analytes according to their solubility.

In contrast to conventional extraction, an increase in extractant volume does not improve extraction efficiency. In some cases, very small solvent volumes are sufficient to provide quantitative extraction; for example, 10 mL of extractant can be used for leaching phenolic compounds from 5 g of soil sample [46].

Temperature is a very important factor that affects extraction efficiency, regardless of the applied technique. During MAE carried out in a closed vessel the

Table 6.5 Selected applications of microwave-assisted extraction in trace analysis

Analyte	Sample/matrix	Extractant	Extraction time (min)
PAHs	Ash, sediments, air, soil	Hexane–acetone, acetone, toluene–water	5–20
PCBs	Sediments, soil	Hexane–acetone, toluene–water	5–10
PCDD/PCDF	Sediments	Toluene	20
Hydrocarbons	Soil, sediments	Acetone	15
Pesticides	Sediments, soil, fish tissue	Isooctane, hexane–acetone, tetrahydrofuran, ethyl octane–cyclohexane	20–30
Imidazole	Soils	Ammonia buffer, pH10	3
Thiazines	Soils	DCM–MeOH (9:1)	20
Drugs	Medicaments, serum, physiological fluids, soil, sediments	Methanol, acetonitrile, chloroform, hexane–isoamyl alcohol	1–15
Methylmercury	Soils	Toluene+HCl	10
Arsenic compounds	Fish tissue	Methanol–water (80:20)	4
Metals	Sediments, plant and animal samples, medicaments, soils	Diluted inorganic acid	10–20

temperature can exceed the solvent boiling temperature. Elevated temperature causes an improvement in extraction effectiveness because of higher desorption of analyte from the matrix, increased analyte solubilization, and better solvent penetration into the sample. The temperature most often applied for extraction of organic pollutants such as PAHs or petroleum-derived hydrocarbons from soils and sediments is around 115–120 °C. Slightly lower temperatures, 80–110 °C, are used during pesticide extraction. For leaching of thermolabile organic constituents, such as aromatic amines or amino acids, lower process temperatures are chosen because of the possibility of analyte decomposition in response to heat.

Other parameters that influence extraction efficiency are time, pressure, and microwave radiation power (closely linked to the temperature of the process) as well as pH and sample mass. All the established parameters are dependent on the kind of sample and its moisture content. In many applications, the efficiency of the extraction process carried out under identical conditions differs significantly for various sample kinds. Therefore, individual adaptation of the procedure is an essential prerequisite for analysis of new materials.

Commercial microwave-assisted systems allow simultaneous processing of several samples in closed vessels, accurately measuring the temperature and pressure. Delivered software permits control and ensures that conditions are appropriate for individual analyses.

There are many published articles on applications of MAE in the analysis of varied materials [48–52]. Selected examples of the use of MAE in trace analysis are given in Table 6.5.

6.3.4 Ultrasound-Assisted Extraction

Sound waves propagate through any elastic transmission medium (solid matter, liquid, or gas) by distribution/dispersion of the mechanical energy of vibrating particles. Ultrasound waves are oscillating sound pressure waves of frequency greater than 20 kHz. Similarly to other sound waves, they spread by propagation of the vibrational energy of particles or molecules. In liquids, mechanical longitudinal waves are generated because of the energy of compression and expansion movements. In solutions, expansion causes hypotension, and thus high-intensity ultrasonic waves create small vacuum bubbles. The bubbles collapse violently during a high-pressure cycle, when they cannot absorb more energy. This phenomenon is termed cavitation. Sudden compression of gases and vapors causes a local increase in temperature and pressure. Implosion of the cavitation bubbles produces liquid jets; the potential energy of expanding bubbles changes into the kinetic energy of liquid jets. Because cavitation bubble size is very small in comparison with solution total volume, local changes in temperature and pressure have no effect on solution parameters. The impact of liquid jets on solid matter is extreme; it can even initiate sonolysis (i.e., cell wall disruption and extraction of intracellular material). Ultrasound is an important aid in sample preparation procedures, facilitating and speeding up extraction of organic and inorganic compounds, slurry dispersion, sample dissolution, emulsification, homogenization, nebulization, cleaning, derivatization, and sample degassing.

Ultrasound-assisted extraction (USE) is an effective method for leaching many analytes from different kinds of samples [52–55]. It is simple, fast, efficient, and inexpensive in comparison with conventional extraction techniques such as solvent extraction in the Soxhlet apparatus. Ultrasound-assisted solid–liquid extraction is an effective and time-saving extraction method. Sonication accelerates the mass-transfer process between two phases. Use of ultrasound results in a reduction in operating temperature, allowing the extraction of temperature-sensitive components. The ultrasound apparatus is cheaper and its operation is easier in comparison with other novel extraction techniques such as MAE.

Two different types of ultrasonic devices are used in laboratories: ultrasonic bath and ultrasonic probe. However, as a result of inhomogeneity of ultrasonic energy distribution in the whole solution and a decrease in power with time, the repeatability and reproducibility of experimental conditions for ultrasonic baths is often unsatisfactory. With ultrasonic probes the energy is focused on a small sample area, which significantly improves cavitation efficiency and, thereby, extraction effectiveness [56].

There are many review articles concerning application of USE in food technology [57] and for isolation of bioactive substances from herbs and other plant materials [58], as well as leaching of heavy metals from environmental and industrial samples [59]. Application of ultrasound during sequential extraction of trace elements significantly shortens the whole procedure; however, for satisfactory efficiency it is necessary to increase the temperature and modify the matrix.

Table 6.6 Selected applications of ultrasound-assisted extraction in trace analysis

Analyte	Sample	Extractant	Extraction time (min)
Phenolic acids	Herbs (*Lamiaceae*)	Ethanol	30
Butyl- and phenyltin	Mussel	Methanol/hexane/toluene, complexing agent tropolone	15–30
Ca, Mg, Mn, Zn	Vegetables	Nitric acid (V)	30; 10
Ag, As, Cd, Cu, Pb	Soil	Aqua regia	9
Mercury compounds	Urine	Dithizone in cyclohexane	3
Fungicides	Soil	Ethyl octane	20
Estrogen, progesterone	Soils	Methanol+acetone (1+1)	15
Isoflavones	Soybeans	Ethanol (50 %)	20
PCBs	Great Blue Heron eggs	Sodium sulfate(VI)/hexane	15
PAHs	Soil	Hexane/acetone, 0.5 M sodium dodecyl sulfateTriton X-100	60
Antibiotics	Animal feed	Methanol/acetic acid	15
Antioxidants	Herbs	Butanone/ethanol/ethyl octane	15/30/45

Another example of ultrasound use is leaching of organic impurities from different kinds of samples. The main analytes of interest are PAHs, which are widespread in soil, sediment, dust, and particulate samples [55]. USE is recommended as a fast, efficient, and direct environmental sample preparation method for determination of PCBs, nitrophenols, pesticides, or polymer additives. Organometallic and biologically active compounds (such as vitamins A, D, and E) present in samples in trace quantities, can be extracted from animal and plant tissues with the aid of ultrasonic wave energy [59]. Table 6.6 presents some typical applications of USE in trace analysis of biological and environmental samples [60].

6.3.5 Sequential Extraction: Fractionation Procedures

Sequential extraction procedures include successive sample treatment with a series of extractants selected on the basis of their ability to dissolve analytes bound to different components of the matrix. The use of sequential extraction procedures simulating natural phenomena, (e.g., acid rain), can deliver detailed information about the origin, mode of occurrence, physiological availability, and mobilization of trace elements. It is also possible to estimate toxicological risk ensuing from the presence of different forms of metals and various phases containing those metals. A

succession of reagents acting more and more destructively leads to isolation of subsequent fraction components with similar chemical properties containing analyzed metals. Sequential extraction procedures are employed most often for environmental samples such as soils, sediments, and waste materials. Typical sequential extraction procedures are based on the five-stage procedure proposed by Tessier and coworkers [61], in which the following components are obtained:

1. Exchangeable (extraction by $MgCl_2$ solution)
2. Bound to carbonates (extraction by sodium acetate solution)
3. Reducible fraction of metals bound to iron and manganese oxides (extraction by hydroxylamine hydrochloride)
4. Oxidizable fraction of metals bound to organic matter (leaching with nitric acid and hydrogen peroxide, followed by ammonium acetate)
5. Residual fraction containing minerals holding metals within their crystal structure (digested with a mixture of HF and $HClO_4$)

During this kind of pretreatment method, samples are centrifuged between stages, usually for 30 min at 1000 rpm. Solution from above the residue is removed by pipette. Residues are washed with deionized water and discarded after subsequent centrifugation. In all the liquid fractions, metals are quantified using spectroscopic methods.

The other popular sequential extraction procedure is the protocol proposed by the Community Bureau of Reference, Commission of the European Community (known as the BCR protocol). The method was proposed on the basis of interlaboratory studies undertaken in order to harmonize conditions for soil and sediment sample analysis. Based on the research data, in 1992 it was stated that application of EDTA or acetic acid solution is appropriate and sufficient for elimination of the bioaccessible fraction of metals from soil samples [62]. In the case of other samples, best results were achieved after application of a three-stage procedure with the following extractants:

1. CH_3COOH solution (bioavailable metals and metals bound to carbohydrates)
2. $NH_2OH{\cdot}HCl$ solution (metals bound to iron and manganese oxides)
3. Mixture of H_2O_2 and CH_3COONH_4 (metals bound to organic matter and sulfides)

Sometimes deionized water is used as first extractant for assessment of water-dissolved element forms. The following have a direct influence on extraction effectiveness and procedure repeatability: extractant chemical properties and selectivity, stage order, extraction time, sample mass to extractant volume ratio, and re-adsorption processes. Other parameters, such as pH, solution concentration, temperature, and phase separation conditions should also be taken into account during the optimization of the extraction process.

Typical sequential extraction procedures employed for soils, sediment, and waste materials are based on the five-stage procedure of Tessier et al. [61]. For plant material (e.g., aquatic moss [3] and spinach [4]), sequential analysis with the use of water, EDTA, petroleum ether, ethyl acetate, butanol, methanol, and

Table 6.7 Comparison of experimental conditions for conventional extraction and processes assisted by ultrasound and microwaves according to the Tessier protocol

Stage	*Fraction*/extractant	Conditions of the leaching process		
		Conventional extraction	Ultrasound-assisted extraction	Microwave-assisted extraction
1	*Exchangeable*/1 mol/dm^3 $MgCl_2$, 8 cm^3, pH = 7	1 h, 25 °C	3 min, 50 % amplitude	30 s, 90 W
2	*Bound to carbonates*/1 mol/dm^3 CH_3COONa, 8 cm^3, pH = 5	5 h, 25 °C	1 min, 50 % amplitude	30 s, 90 W
3	*Reducible*/0.04 mol/dm^3 $NH_2OH{\cdot}HCl$/25 % (w/w) CH_3COOH, 20 cm^3	6 h, 96 °C	7 min, 50 % amplitude	30 s, 90 W
4	*Oxidizable*/0.02 mol/dm^3 HNO_3/ 30 % H_2O_2 + 30 % H_2O_2 + 3.2 mol/ dm^3 CH_3COONa, 3 cm^3/5 cm^3 + 3 cm^3 + 5 cm^3	2 h, 85 °C; 3 h, 85 °C; 30 min, 25 °C	7 min, 50 % amplitude; 2 min, 50 % amplitude	30 s, 270 W; 10 s, 270 W

Table 6.8 Fractionation of elements from oil fly ash (modified BCR protocol)

Element	Ashed material	Water-removable fraction (%)	Fraction I (%)	Fraction II (%)	Fraction III (%)	Residual (%)
Zn	Petroleum	30.8	1.1	0.1	0.1	60.6
	Heavy oil	19.2	0.7	0.3	0.7	79.2
P	Petroleum	26.8	0.3	42.5	9.2	21.2
	Heavy oil	24.7	0.3	<0.1	1.3	73.7
Cd	Petroleum	15.3	0.3	2.7	0.1	81.7
	Heavy oil	41.1	0.5	1.3	<0.1	57.2
Fe	Petroleum	10.8	0.5	4.0	<0.1	84.7
	Heavy oil	43.4	0.6	1.6	<0.1	54.4
Co	Petroleum	31.8	0.8	0.1	0.5	66.9
	Heavy oil	51.3	0.8	0.3	0.8	46.7
Ni	Petroleum	20.6	0.6	<0.1	0.2	78.6
	Heavy oil	34.3	1.2	0.2	1.7	62.6

hydrochloric acid was carried out to isolate organic compounds binding trace elements. Frequently, instead of the conventional sequential extraction procedure (shaking and heating in a water bath), ultrasound or microwave energy are applied, which significantly shorten the time of experiments. Table 6.7 compares experimental conditions of the conventional Tessier procedure with similar procedures assisted by ultrasound and microwave energy [63, 64].

Based on real environmental conditions, several procedures simulating the influence of the environment on different materials have been developed. Examples of fractionation of elements in flay ash produced during combustion of fuel oil are shown in Table 6.8 [65, 66].

6.3.6 Enzymatic Extraction: Biofractionation

Enzymatic extraction procedures are employed to assess the bioaccessibility of microelements and other essential nutrients and active compounds, usually in solid and liquid food products [67–70]. The method is also applied to soil samples in order to estimate the quantity of elements released in the human digestive system after ingesting soil. Application of in vitro enzymatic extraction procedures leads to determination of the amount of compound released from the matrix during gastrointestinal digestion in the human organism. Extraction carried out using simulated gastric and intestinal fluids (i.e., solutions containing digestive enzymes) gives information on food constituent fractionation and allows estimation of nutrient bioavailability. The first part of the human digestive tract is the mouth, where saliva is secreted. Saliva contains amylase, which begins to break down starch and glycogen, and salivary lipase, which is capable of starting fat digestion. In most of the commonly applied in vitro enzymatic extraction procedures this stage is omitted because of the short time that foods remain in the mouth. The next digestion stage takes place in the stomach. Gastric digestion is simulated by the use of a solution with a composition similar to that of the gastric juices, containing pepsin, hydrolyzing proteins, and hydrochloric acid as pepsinogen activator. Optimal pH for pepsin functioning ranges from 1 to 2. Usually, procedures simulating gastric digestion last from 2 to 4 h. Intestinal digestion is mimicked in the laboratory by extraction with solutions containing pancreatic enzymes. Most often amylase, lipase, trypsin, and chymotrypsin (or pancreatin, which is a mixture of the previously listed enzymes) are chosen. Amylase catalyzes the hydrolysis of starch disaccharides and trisaccharides. Lipase acts on dietary fats, converting triglycerides to monoglycerides and fatty acids. Trypsin and chymotrypsin are proteases that hydrolyze peptide bonds, breaking down proteins into smaller peptides, namely amino acids. Extractants, similarly to intestinal juice, also contain bile salts, mucus, and substances to neutralize hydrochloric acid coming from the stomach. Optimum pH, at which pancreatic enzymes have maximal activity, ranges from 7 to 8. Samples are incubated in the intestinal juice substitute for 1–6 h, most often 2 h.

In vitro digestion procedures based on human physiology can reproduce in the laboratory, in one step or a sequence of stages, the food digestion processes taking place in the mouth, stomach, and small intestine. Most often, procedures simulating two digestion stages (gastric and intestinal digestion) are applied. Simulations of the processes taking place in the human digestive system are often carried out in simple laboratory glass vessels; however, complex, unmanned models involving chewing, peristaltic movement, and nutrient absorption have also been constructed. Extractant solutions containing enzymes specific for individual parts of the digestive tract are used. Acidity of the solutions for optimal enzyme activity is adjusted by acid or base additions (usually HCl and $NaHCO_3$).

All enzymatic extraction procedures are conducted at 37 °C, which is similar to normal body temperature. Simulation of gastrointestinal movement and mixing of food during enzymatic hydrolysis is reproduced by shaking, stirring (mechanically

Table 6.9 Examples of enzymatic extraction in element trace analysis

Analyte	Sample	Enzyme and type of digestion	pH
Ag, As, Cd, Cu, Fe, Mg, Pb, Zn	Mussel, dogfish muscle, dogfish liver	Pronase/lipase; intestinal digestion	7
As speciation forms	Supplements for children	Trypsin/pankreatin; intestinal digestion	7
Se speciation forms	Supplements enriched with selenium	Pepsin; gastric digestion	2
Se	Yeast, oyster, mussel	Pronase; intestinal digestion	7
As speciation forms	Fish, dogfish	Trypsin; intestinal digestion	7
Al, As, Cd, Cr, Cu, Mn, Ni, Pb, Zn	Shellfish	Pepsin; gastric digestion	2
		Pancreatin/amylase	9 (As, Cd, Cu, Fe, Ni)
		Pancreatin/lipase; intestinal digestion	6 (Al, Cr, Mn, Pb, Zn)
Cu, Pb	Wine	Pepsin; gastric digestion	3.5
		Pancreatin+amylase; intestinal digestion	7.4
As	Soil	Pepsin; gastric digestion	2.5
		Pancreatin; intestinal digestion	7.0

or magnetically), or swirling the samples, usually in a water bath. Reduction in the time needed for enzymatic hydrolysis to 30 min was achieved using ultrasonic energy [71]. In vitro enzymatic extraction efficiency is directly influenced by temperature, extractant acidity (affecting enzyme activity), enzyme concentration, and hydrolysis/extraction time.

Enzymatic extraction carried out using in vitro models of the gastrointestinal tract is cheaper, faster, more reproducible, and ethically easier than the alternative of studies involving people and animals. Estimation of nutrient bioavailability (including trace elements, both essential and toxic) is particularly important for nutritionists, pharmacists, and toxicologists. Application of sequential procedures allows analyte fractionation (metals usually), but enzyme selectivity allows leaching of certain speciation forms of the determined elements. Table 6.9 gives examples of application of enzymatic extraction procedures for trace element analysis and speciation analysis [71, 72].

6.3.7 Cloud Point Extraction

Cloud point extraction (CPE), also called micelle-mediated extraction, is based on the phase separation behavior exhibited by aqueous solutions of some surfactants, usually nonionic, anionic, or zwitterionic. CPE has been recognized as an alternative approach to conventional liquid–liquid extraction because of a number of advantages: low cost, environmental safety, short analysis time, simultaneous pretreatment of a large number of samples is possible, and high recovery for a wide variety of analytes. The cloud point phenomenon occurs when aqueous solutions of surfactants at concentrations above the critical micelle concentration (CMC) associate to form molecular aggregates (micelles) upon modification of temperature or pressure, or introduction of a suitable additive. Another series of structures can also be formed, depending on the surfactant characteristics, concentration, and temperature. Regular micelles have a nonpolar core (hydrophobic tail) inside and an extended polar layer (hydrophilic head) outside. The principles and theoretical background, as well as analytical aspects, regarding use of surfactants have been well documented [73–75].

In aqueous solutions the micellar assembly structure allows sparingly soluble or water-insoluble chemical species to be solubilized, because they can associate and bind to the micelles. The interaction between surfactant and analyte can be electrostatic, hydrophobic, or a combination of both [76]. The solubilization site varies with the nature of the solubilized species and surfactant [77]. Micelles of nonionic surfactants demonstrate the greatest ability for solubilization of a wide group of various compounds; for example, it is possible to solubilize hydrocarbons or metal complexes in aqueous solutions or polar compounds in nonpolar organic solutions. As the temperature of an aqueous nonionic surfactant solution is increased, the solution turns cloudy and phase separation occurs to give a surfactant-rich phase (SRP) of small volume containing the analyte trapped in micelle structures and a bulk diluted aqueous phase. The temperature at which phase separation occurs is known as the cloud point. Both CMC and cloud point depend on the structure of the surfactant and the presence of additives. Table 6.10 gives the values of CMC and cloud point for the surfactants most frequently applied in the CPE process.

In the case of nonionic surfactants, an increase in temperature promotes a dehydration of the poly(oxyethylene) chains and growth of aggregates. Phase separation occurs as a rule in a narrow range of temperature, and the phenomenon is reversible. The ratio of the volume of organic to aqueous phase can be very small

Table 6.10 Critical micelle concentration (CMC) and cloud point temperatures for selected nonionic surfactants

Surfactant	CMC (mM)	Cloud point (°C)
Triton X-100	0.17–0.30	64–65
Triton X-114	0.20–0.35	23–25
PONPE 7.5	0.085	5–20
Brij-30 ($C_{12}E_4$)	0.02–0.06	2–7
Brij-56 ($C_{16}E_{10}$)	0.0006	64–69
Genapol X80 ($C_{12}E_8$)	0.05	75

(0.007–0.04). In consequence, the analyte contained inside micelles or connected with surfactant aggregates can be separated and preconcentrated in the organic-rich phase. Such a method of separation of analyte has many advantages. Among others, it is experimentally simple, inexpensive, and the reagents used are nontoxic and less hazardous than organic solvents. CPE is recognized as a method in agreement with the principles of green chemistry.

The first application of CPE was proposed by Watanabe and coworkers [78] for the separation and preconcentration of metal ions. From then, the methodology has gained increasing attention both for the determination of different metal ions in various samples as well as for extraction of organic analytes. The scheme of CPE that can be found in review papers [73–82] is as follows: In the first stage, a surfactant is added to the analyte solution to a concentration above the CMC. Next, the solution is heated above the cloud point in water, ultrasonic bath, or microwave oven until phase separation. The optimal equilibrium temperature occurs when the temperature is 15–20 °C higher than the cloud point. After spontaneous gravity settling or centrifugation, the analyte remains entirely in the organic phase. If needed, the procedure can be repeated by addition of surfactant to the aqueous phase [83]. To increase the viscosity of the SRP and to ensure a better grip to the vessel walls, the mixture is cooled in an ice bath (with NaCl, acetone). Removal of the aqueous phase is carried out by decanting or using a pipette or syringe pump. Total water removal is possible by additional volatilization in a stream of argon, helium, or nitrogen. In the case of thermally stable compounds such as metal complexes, heating above 100 °C in air can be applied. Reduction of the final organic phase volume to the initial volume determines the preconcentration factor, which is defined as the ratio of analyte concentration in the SRP to that in the original aqueous solution before extraction. In order to facilitate instrumental analysis of the final solution it is necessary to decrease the viscosity of the SRP by adding appropriate diluting agent. Small amounts of water, methanol, ethanol, acetonitrile, or inorganic acids are used for this purpose, depending on the detection system used.

Coupling CPE to flow injection analysis (FIA) has also been exploited in both on-line and off-line configurations [73, 84]. The use of FIA to introduce the SRP into various analytical devices facilitates the dissolution of the SRP in small volumes (increased preconcentration factor), alleviating reproducibility problems [73].

The optimal range of surfactant concentration at which a quantitative extraction efficiency is achieved is narrow and should be established individually for each process. As a rule, CPE is conducted at elevated temperature, which beneficially affects kinetics and causes partial dehydration of micelles, thus increasing the ratio of the phase volumes (V_{aq}/V_{org}).

Several parameters have to be considered and optimized to achieve maximum efficiency and preconcentration: type and concentration of surfactant, additives (ionic strength), pH, equilibration temperature and time, and centrifugation conditions. The ionic strength affects the facility of phase separation and the cloud point and should be established individually.

Table 6.11 Examples of application of cloud point extraction in trace analysis

Analyte	Sample	Detection method
Organic compounds (PAHs, phenol and derivatives, PCBs, dibenzofurans and dibenzodioxins, pesticides, humic compounds, phthalic acid esters, amines, drugs)	Natural waters, waste, serum, biological fluids, plant and animal samples, sediments	LC, HPLC
Metals ions (Ag, Al, As, Au, Ba, Be, Bi, Cd, Co, Cr, Cu, Dy, Er, Fe, Ga, Gd, Hg, In, La, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Ru, Se, Tl, U, V, Zn)	Natural waters, biological fluid, brines, waste, fertilizers, hair	AAS, ICP-OES, ICP-MS, CE, spectrophotometry, spectrofluorometry
Speciation analysis (Cr, Cu, Fe, Hg, Mn, Sb, Se, Sn)	Synthetic samples, water, wine	AAS, ICP-OES, ICP-MS, spectrophotometry

AAS atomic absorption spectroscopy, *ICP-OES* inductively coupled plasma optical emission spectrometry, *ICP-MS* inductively coupled plasma mass spectrometry, *CE* capillary electrophoresis

For organic molecules, pH is one of the most crucial factors regulating the partitioning of the target analyte in the micellar phase [73, 76, 79]. Especially for ionizable species, such as phenols and amines, maximum extraction efficiency is achieved at pH values at which the uncharged form of the target analyte prevails. In the case of elemental analysis, the metal can be in ionic form or in hydrophobic chelates that are produced after reaction under appropriate conditions. pH is a crucial parameter for quantitative complex formation and plays an important role in improving the extraction efficiency of metals without addition of a chelating agent, because it affects the overall charge of the analyte and the formation of complexes between the metal and the surfactant polyoxyethylene groups [77, 80–82]. Optimization of other experimental parameters such as ligand concentration, surfactant type and concentration, ionic strength, and solvent type and volume should also be conducted. For separation of metal ions in the form of hydrophobic chelates, the most frequently used are carbamates, pyridylazo, quinoline and naphthol derivatives, dithizone, 8-hydroxyquinoline, and *O*,*O*-diethyldithiophosphate [73, 80, 85]. Table 6.11 presents some examples of CPE application in trace analysis of organic and inorganic species.

6.3.8 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) utilizes the properties of supercritical fluids for extraction of analytes from solid samples. A supercritical fluid (SCF) is a substance above its critical temperature and pressure, when it is between the typical gas and liquid state. Low viscosity and near-zero surface tension and heat of vaporization allow SCFs to penetrate into solids more rapidly than liquid solvents, which leads to more favorable mass transfer. The density of an SCF is close to the liquid density,

Table 6.12 Critical parameters for selected substances used in supercritical fluid extraction

Substance	Critical temperature (°C)	Critical pressure (atm)	Critical density (kg/dm^3)
CO_2	31.3	72.9	0.47
N_2O	36.5	72.5	0.45
SF_6	45.5	37.1	0.74
NH_3	132.5	112.5	0.24
H_2O	374	227	0.34
n-C_4H_{10}	152	37.5	0.23
CHF_3	25.9	46.9	0.52

whereas its viscosity is close to the gas viscosity. SCFs can diffuse through solids like a gas and dissolve materials like a liquid. Additionally, close to the critical point, small changes in pressure and/or temperature result in large changes in density. This allows many SCF properties to be precisely adjusted and remarkable selectivity to be achieved.

The critical parameters of some substances used as solvents or SCFs are shown in Table 6.12. The majority of analytical SFEs use supercritical CO_2 as extracting agent because of its preferred low values of critical pressure and temperature [86, 87]. Moreover, it is nontoxic, colorless, odorless, nonflammable, inexpensive, and easy available. Because of its nonpolar nature, CO_2 cannot be used for dissolving polar molecules. For extraction of polar compounds, N_2O or $CHClF_2$ are more suitable in the supercritical state but in routine analyses they are relatively rarely used [88]. The solubility of polar compounds in supercritical CO_2 and the selectivity of the process can be increased by adding small quantities (1–10 %) of other polar solvents, called modifiers or co-solvents. All SCFs are completely miscible with each other, so a single phase can be guaranteed for a mixture if the critical point of the mixture is exceeded. Commonly used modifiers for extraction of compounds containing oxygen are ethanol, methanol, acetone, tetrahydrofuran, and 2-propanol. Addition of acetonitrile is usually applied for extraction of nitrogen compounds whereas formic acid is popular for extraction of acids. For sulfur-containing compounds, CS_2, SO_2, or SF_6 can be used as modifiers [1]. MeOH, HOAc, and aniline as acidic/basic modifiers greatly enhance the extraction of polychlorinated biphenyls (PCBs). Modifiers such as toluene, diethylamine, and CH_2Cl_2 are the best modifiers for SFE of high molecular weight PAHs [89, 90]. Direct extraction of metal ions by SCFs is inefficient as a result of the charge neutralization requirement and the weak solute–solvent interactions. However, if metal ions are converted into neutral metal chelates, their solubility in supercritical CO_2 increases. This is realized by modification of SCFs by the addition of complexing agents [90, 91].

The most suitable samples for SFE are finely powdered solids with good permeability, such as soils, sediments, and dried plant and biological materials. Extraction of wet or liquid samples and solutions is more difficult [56, 90]. The basic components of SFE include a tank of CO_2, a high-pressure pump, an extraction cell, a heating oven, a flow restrictor controlling the pressure of the SCF, and a

Table 6.13 Examples of application of supercritical fluid extraction in trace analysis

Analyte	Matrix/sample	Extractant
Dioxins	Soils, marine sediments, plants, sludge, urban and industrial waste	CO_2+toluene
PAHs		CO_2+toluene, CO_2+CH_3OH
PCBs	Soils	CO_2+CH_2Cl_2
Herbicides, pesticides	Soils, fruit and vegetables and their preserves, animal tissues, food	CO_2+methanol, acetone, ethanol, water
PAHs	Soils, sediments	
Drugs, narcotics	Blood, urine, soft tissues, hair	CO_2, CO_2+CH_3OH, NH_3
Metal–organic compounds, speciation forms: As, Hg, Sn, Pb	Solutions, sediments, food, shells, fish, sand, dust	CO_2+HCOOH, CO_2+CH_3OH
Heavy metals	Water, soil, ash, wood, sediments, sand, dust	CO_2+complexing agents

collection vessel. Modifier is supplied by an individual delivery system [1]. The sample is put into the extraction vessel and heated.

The extract is collected by depressurization on a column packed with a solid sorbent, in a vessel containing the appropriate solvent, in a collection device connected to a chromatograph, or on combined solid phase–solvent traps [92]. For extraction of volatile compounds, such solvents as acetone, CH_2Cl_2, methanol, or liquid nitrogen are used. Silica gel columns are the most popular way of trapping solids. In this case, the selectivity of the process can be improved by selective elution of the sorbent [88, 92]. SFE can be conducted in a static mode in which sample and solvent are mixed and kept for a user-specified time at a constant pressure and temperature, or in a dynamic mode where the solvent flows through the sample in a continuous manner [56]. The extracted analytes can be collected into an off-line device or transferred to an on-line chromatographic system for direct analysis.

SFE is an efficient and fast extraction technique that fits well with green chemistry strategies. The broadest applications of SFE can be found in food, environmental, and pharmaceutical analysis, in industrial and biomedical laboratories, and in speciation analysis [86, 88, 93–97]. Table 6.13 presents selected examples of the use of SFE in trace analysis.

6.3.9 QuEChERS Technique

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is an acronym used as the name of a sample preparation procedure, generally based on a combination of solvent extraction of analytes from a sample and dispersive SPE applied for cleaning an extractant.

The QuEChERS procedure consists of a number of simple analytical operations and is little susceptible to errors. Two steps can be generally distinguished. The first step (obtaining raw extract) involves extraction of analytes from homogenized sample with organic solvents, usually acetonitrile, followed by addition of salts (most often $MgSO_4$ and NaCl). Salts are added for salting out the water from an extract (i.e., phase separation). Sometimes pH adjustment by buffer addition is indispensable for analyte partitioning. During the second step, the final extract is obtained using a cleaning procedure carried out by addition of sorbent and $MgSO_4$. The final extracts are analyzed by GC and/or LC methods and are preferably performed in a direct way.

Universal kits, developed and widely commercially available for the QuEChERS procedures, provide excellent recoveries and reproducibility. The complete commercial QuEChERS kits are of great advantage in standardization of this technique and in assuring high quality of analytical achievements, compatible with current methodologies recommended by both the Association of Analytical Communities (AOAC) and European Committee for Standardization (CEN).

The QuEChERS method was invented and described for the first time in 2003 by Anastassiades et al. [98] as a fast, simple, inexpensive, and convenient preparation procedure for fruit and vegetable samples used for pesticide multiresidue analysis. Currently, this methodology is used for determinations of pesticides, pesticide residues, and other compounds of environmental concern such as phenol derivatives, perfluorinated compounds, and chlorinated hydrocarbons; pharmaceutical compounds in food and agricultural matrices; and environmental samples such as soil, sediments, and water (see for example [99–102]).

6.4 Summary

Current and future trends in analytical extraction methods and their applications are result of the challenges and high requirements facing modern analytical chemistry, especially in the area of trace analysis. Therefore, the trends and developments in extraction analytical techniques must take into account the need to determine analytes at significantly lowered limits of quantification; assure high quality and reliability of measurements by validation and traceability; comply with ecological and economic requirements for a decrease in the use of energy and materials (especially organic solvents); and use miniaturized analytical devices operated and controlled by sophisticated electronic systems. Numerous microextraction techniques of different kinds are very good examples of these trends. Assistance of the extraction process by microwave and ultrasound energies, high pressure, and solvent acceleration have become widespread and promising methods that can lead to higher efficiency and shortening of procedure time.

Especially promising and of great important are modifications of the SPE technique and introduction of solvent-free techniques, applicable for some groups of analytes. This has been made possible by the successful development and

implementation of new sorption materials with special, predicted, and programmed properties. A typical tendency also observed is hyphenation of different analytical methods and techniques and their combination with chromatographic methods (GC, HPLC, nanoHPLC). New extraction techniques can be implemented with the utilization of miniaturized analytical devices handled and controlled by sophisticated electronic systems.

Introduction of modern technical solutions enables miniaturization and automation of extraction procedures and their implementation for in situ analyses. The most spectacular progress can be observed in miniaturization and automation of the extraction reactors, leading to commercial devices applied for in situ analyses and mobile advanced instruments.

Progress in the fractionation and speciation analysis of elements with the use of extraction is still far from needs and expectations, undoubtedly as a result of the insignificant/insufficient number and small diversity of available reference materials.

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Part II
Application of Trace Analysis

Chapter 7
Trace Analysis of Selected Organic Compounds

Iwona Rykowska, Wiesław Wasiak, and Bartosz Kowalski

7.1 Introduction

Trace analysis of organic compounds is primarily used in the detection and determination of harmful substances of natural origin (e.g., mycotoxins) as well as those that are the undesirable result of human activity, especially industrial and agricultural. Of the latter, the subject of interest could be either intentionally produced compounds (pesticides, flame retardants, chemical weapons, etc.) or unwanted impurities released in an uncontrolled manner in technological processes or from improper combustion of fuels and waste materials [1, 2].

Main areas of application of trace analysis include:

- Environmental chemistry (analysis of the circulation of matter in nature)
- Biochemistry (gene analysis, proteomics, metabolomics, etc.)
- Ecotoxicology (e.g., study of bioaccumulation of trace elements)
- Medical diagnostics
- Diagnosis of food products (analysis of the composition and impurities)
- Material engineering (especially in the field of ultrahigh-purity materials, such as those used in crystallography and the semiconductor industry)

Analyses in biochemistry and medical diagnostics are of primary concern because even trace amounts of certain substances can have a big impact on the overall health of living organisms. Of particular importance is detection and identification of carcinogenic and estrogenic substances (being a main cause of

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cancer). According to the classification introduced by Kundson [3], the main causes of cancer are the following:

- Genetic predispositions
- Environmental factors
- Undefined factors

It should be noted that the concept of the natural environment should be understood as not only comprising water, air, and soil, but also food, way of life, occupational exposure, drugs, and all aspects of human interactions with the environment. It is assumed that the vast majority of cancer cases can be prevented by avoiding exposure to potential carcinogens and by changing the way of life.

7.2 Carcinogens

A carcinogen is a chemical compound that causes a change in the original genetic information (mutation), which contributes to the development of cancer.

Known substances with proven carcinogenic effects belong to different chemical groups, and the construction and properties common to all the features conditioning carcinogenic properties cannot be identified. The basic set of compounds with proven carcinogenic nature includes:

- Inorganic compounds such as salts of arsenic, chromium, and nickel, which go beyond the scope of this review
- Organic compounds such as benzene, 2-naphthylamine, vinyl chloride, and polycyclic aromatic hydrocarbons
- Complex substances such as soot, tar, and mineral oils
- Natural substances such as aflatoxin, mitomycin C, phorbol esters, and nitrosamines

In food, we most commonly observe such carcinogens as aflatoxins, dioxins, mycotoxins, *N*-nitrosamines, and other organic compounds.

Aflatoxins are soluble in water, readily pass through the membranes and tissues of plants and animals, including the skin. These compounds accumulate in the body, leading to functional disorders and further diseases and death.

Dioxins compose a group of chlorinated aromatic compounds whose molecules exhibit exceptionally high thermal stability and chemical resistance to oxidation and biological degradation processes. The full name of these compounds is polychlorinated dibenzo-*para*-dioxins. These substances belong to a group of highly toxic active compounds that are artificially produced by man. Dioxins consist of two benzene rings connected by two oxygen atoms, and from one to eight chlorine atoms attached to the benzene rings. The common name “dioxin” refers to all possible chlorinated oxanthrenes (dibenzo-1,4-dioxins). Because of similar toxic properties and their presence in nature, the group of dioxins also includes polychlorinated dibenzofurans and polychlorinated biphenyls (PCBs).

Mycotoxins are products of secondary metabolism of various kinds of filamentous fungi (molds) that produce them as a by-product of metabolism or for defensive purposes. Mycotoxins can have strong toxic, mutagenic, or teratogenic properties and are found in a wide range of agricultural products under different environmental conditions.

7.3 Compounds Showing Estrogen-Like Activity

7.3.1 Estrogens

Endocrine disrupting compounds (EDCs) comprise a large group of compounds that potentially interfere with natural biological functions by blocking hormone action through imitation, change, or even destruction of their natural activity in living organisms. These compounds can include organochloride pesticides and organophosphate pesticides, softeners (plasticizers), phthalates, PCBs, polybrominated diphenylethers (PBDEs), brominated flame retardants (BFRs), perfluorocarbons, antibiotics, nonsteroidal anti-inflammatory agents, cardiovascular drugs, hormonal agents, and surface active compounds and their metabolites (phenols). Most of these are ubiquitous and extremely persistent in the environment, bioaccumulate in the food chain, and can be stored in adipose tissue, where they are slowly metabolized and excreted [4].

EDCs are compounds largely of natural origin. Increasingly, however, similar synthetic substances are being produced by humans and introduced into the environment in various ways. These substances are called environmental estrogens, or xenoestrogens. Estrogen-active compounds are a group of compounds of particular carcinogenic effect [5]. An estrogenic compound is one that mimics natural estrogen by binding to its receptor. Many of the compounds produced by the human body show estrogenic activity. Synthetic compounds include some organochlorine insecticides, phthalates, and nonylphenols. Although the functions of EDCs have been known for many years, only recently have these compounds become of more interest and one of the most controversial issues of environmental pollution.

Xenoestrogens, irrespective of their origin, have the ability to interact with the endocrine system, disturbing its operations in a manner characteristic for a given estrogen. Development and dissemination of estrogen-based drugs (contraceptives, hormone replacement therapy) has resulted in the release to the environment of large amounts of these substances. It is suspected that these substances are responsible for causing side effects in mammals such as impaired fertility, lack of masculine features, etc. These phenomena are increasingly common among wildlife in marine and inland waters. Environmental estrogens present in drinking water are also responsible for causing fertility problems in men and disorders of gender in the human fetus. These compounds are present in plastic bottles, toys for children,

cosmetics, food packaging, natural waters polluted by urban wastewater, water from swimming pools, and processed foods (meat, soy products).

Given the complexity of the endocrine system, the mechanisms of action of xenobiotics causing dysfunction are extremely complex and not fully understood. The effects can be particularly dangerous in utero and childhood, as a result of the lack of developed mechanisms and feedback for regulating the operation, synthesis, and excretion of hormones. This can lead to irreversible changes, which manifest themselves sometimes with a long delay. The relationship between exposure to EDC and the health of living organisms is widely discussed in the literature. It seems that there is a risk that exposing an adult to an EDC can cause temporary or permanent hormonal disturbances, which in turn can lead to permanent damage to the fetus. A fetus, because of its small size, high dynamics of growth processes, and reduced ability to detoxify harmful substances is particularly sensitive to EDCs [6]. Most of the EDC is able to pass readily through the placenta and enter fetal circulation. In recent decades there have been many studies of the transmittance of EDCs and assessment of their content in the cord blood serum and maternal adipose tissue [7–20]. Intensification of fat reserves during pregnancy and while breast-feeding means that a fetus, and later an infant, can be significantly exposed to high levels of EDC [21]. It was found that the main effect of prenatal exposure to EDC is a decrease in fetal birth weight, premature birth, psychomotor retardation, and altered cognitive function [22–27].

Food is a primary source of exposure to xenoestrogens. To a lesser extent, they enter the body through the respiratory system or the skin. After reaching plasma they bind to proteins or remain there unbound. As a result of metabolic transformation xenoestrogens can be converted to non-active substances and/or excreted in urine or by other routes. Some are transported to the target tissues and organs and produce a particular effect by combining with a receptor or by other mechanisms. Some compounds have hormonal activity in the body only after conversion to an active metabolite, which could also lead to changes in the activity of the compound. If a given xenobiotic is a persistent compound, it can accumulate in tissues and organs (e.g., in adipose tissue), where it can then be slowly released into the body. In many cases, the duration of its action can be the entire life of the organism exposed.

7.3.2 Bisphenol A

In the last few years, special attention has also been paid to the exposure of humans to bisphenol A (4´-dihydroxy-2,2-diphenyl, BPA; Fig. 7.1), well-known for its estrogenic properties. BPA [28] is commonly used as an industrial plasticizer and is found in paints, flame retardants, unsaturated polyester resins, plastic food packaging, containers for water, infant feeding bottles, and foils used for food storage. Several studies have confirmed the presence of BPA, at a detectable level, in a wide variety of food containers [29–31]. Views on the potential danger

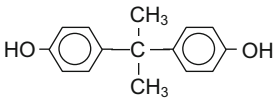


Fig. 7.1 Bisphenol A: 2,2-bis-(4-hydroxyphenyl)-propane

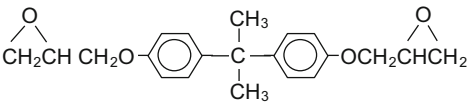
of BPA are varied. It is believed that hydrolysis of the ester bonds present in polymers and nonpolymeric monomers based on BPA is responsible for the widespread contamination by this group of compounds. Nevertheless, in the literature information can also be found about the harmlessness of BPA. According to these reports, BPA cannot be regarded as an important biological pollution because it is relatively quickly metabolized and excreted from the body. On the other hand, there are also reports in the literature demonstrating that the transport of BPA is carried out by the placenta, where only part of it is metabolized and excreted. The rest remains in women, exposing both the mother and fetus to long-term risk of exposure to BPA and related negative consequences [32–34]. It is necessary to realize that, even at very low concentrations, BPA shows potential estrogenic properties [35, 36]. Numerous biochemical and toxicological studies have confirmed that bisphenol A has estrogenic properties and agonist activity for the estrogen receptor. Recent studies indicate that there is complete basis for including BPA in the group of xenobiotics that disturb the balance of the endocrine system in animals and humans (i.e., hormonally active agents).

World production of BPA (started in 1957 in the USA) in 2001 was estimated at about 2.5 million tons. Products containing BPA have been applied in many areas of life, for example, as varnish covering metal boxes and cans (from the inside) and other vessels used to store water, food, and pharmaceuticals. In addition, polycarbonate plastic is commonly used to produce medical equipment (e.g., for dialysis and blood oxygenation), baby bottles, and tableware such as plates and cups. Polycarbonates are so widely used because of their characteristic lightness, durability, high tensile strength and high modulus, high melting point, and high glass transition temperature. Polycarbonates absorb little water, are resistant to light and other atmospheric agents (e.g., high temperature), and show good electrical insulating properties.

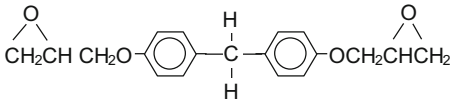
For many years, BPA has been treated as a nontoxic to living organisms. In the early 1990s, detection of BPA in the environment and also in drinking water and food packaging caught the attention of many researchers. As a result, in 1996, BPA was classified by the European Commission as an extrinsic substance adversely affecting the health of the body and offspring.

7.3.3 Bisphenol-A Derivatives

Apart from pure bisphenol A, two of its derivatives, bisphenol-A diglycidyl ether (BADGE) and bisphenol-F diglycidyl ether (BFDGE), are very important for industry. Figure 7.2 shows the structural formulae of these two compounds.



BADGE



BFDGE

Fig. 7.2 Structural formulae for BADGE and BFDGE

BADGE is the product of reaction between bisphenol A and epichlorohydrin. It is the main compound used in industry for producing epoxy resins, which, as previously stated, are widely used as coatings for food containers. It is estimated that 75 % of the epoxy resins used today are derived from BADGE. In addition, this compound is used as a coating for cans and other vessels used for food storage. BADGE has been classified as a mutagenic compound (i.e., one that causes changes in DNA, mutations). Mutagenic substances can also cause cancer with a certain probability, but mainly they cause genetic diseases in the offspring of an exposed person (all carcinogens operate on the principle of mutation, but these mutations are not always inherited by the offspring). This is because such substances cause changes in the DNA contained in the germ cells. If the amount of damage exceeds the capacity of corrective mechanisms of the testicle or ovarian cells, germ cells can occur with an altered genetic record. This causes a variety of diseases and defects that may appear in later generations.

The second compound mentioned above, BFDGE, is used as a raw material for the production of epoxy resins and as a coating for covering the inside of food cans.

Following the literature devoted to the determination of bisphenol A and its derivatives, it can be concluded that the number of publications on this subject is increasing every year. Most of these compounds are determined by gas chromatography (GC) and high performance liquid chromatography (HPLC) combined with mass spectroscopy (MS) techniques. Most works describe the determination of bisphenol A in environmental samples [37, 38] such as surface water, sewage, river water, groundwater, and in food samples stored in cans and packages made of polycarbonate. Tap water having contact with polycarbonate bottles for infants, and human blood, were also studied for the presence of this compound. Table 7.1 shows the level at which BPA is present in the environment in some products.

The literature also describes the ability of BPA, BADGE, and BFDGE to pass from polymers to food, particularly under elevated temperature and improper use of polymeric containers and packaging. Over the past few years, numerous organizations for protection of the environment and global industry have explored the potential low level of BPA migrating from polycarbonate products to food and beverages. These studies consistently demonstrate that the migration of bisphenol A to food is low, generally less than 5 parts per million (<5 ppm), provided in

Table 7.1 Bisphenol A concentration in selected products

Food	0.07–0.42 ppb
Baby food	0.1–13.2 ppb
Powdered milk	~45 ng/g
Water	0.016–0.5 μg/L
Blood serum	0.46–0.56 ppb
Cord blood	0.46–0.62 ppb

accordance with the manufacturer's instructions concerning products made of polycarbonate. The results of studies estimate that the reception of polycarbonate BPA migrating from food is less than 0.0000125 mg/kg of body weight per day. This level is 240,000 times lower than the maximum level of 3 mg/kg. Migration studies therefore show low levels of polycarbonate BPA entering into food.

The Scientific Committee on Food (SCF), which is an independent advisory committee of the European Commission, estimated safe levels for BPA that enters into food. The SCF fixed the tolerable daily intake (TDI) for bisphenol A at 0.01 mg/kg of body weight per day, after comprehensive and exhaustive analysis of all the scientific facts covering all aspects of toxicity. The food migration limit set by the European Commission for BPA is 3 mg/kg, whereas for BADGE and BFDGE it is 1 mg/kg of body weight.

7.3.4 Alkylphenols

Among the compounds belonging to the EDC group, a separate class, most often identified as substances responsible for the observed estrogenic effect, is related to steroids and alkylphenol ethoxylates (APELs). Alkylphenols were brought into service in the 1940s. They have been used as ingredients of paints, herbicides, pesticides, certain nonionic detergents, cosmetics, plastic containers, composite materials, and as a medium for the degreasing and surface treatment of textiles, leather, etc.

4-Nonylphenol (4NP) and octylphenol (4OP), whose structural formulae are shown in Figs. 7.3 and 7.4, are metabolites of microbial degradation of ethoxylated nonylphenol and have been found in both aqueous environments and wastewater treatment plants [3]. The compound 4-*tert*-nonylphenol (4tNP) (Fig. 7.5) is still widely used in industry. This compound, in addition to its estrogenic activity, also shows toxic properties to living organisms, including humans.

These compounds are released, for example, from plastic materials and enter surface waters, where they are subject to accumulation. The concentration of these xenoestrogens in surface water is at the level of micrograms, and even milligrams, per cubic meter. The highest content of nonylphenol was recorded in England (180 mg/m^3) and octylphenol in Wales (13 mg/m^3). Table 7.2 summarizes the determined concentrations of xenoestrogens in environmental samples (surface waters).

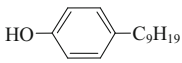


Fig. 7.3 4-Nonylphenol (4NP)

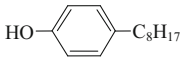


Fig. 7.4 4-Octylphenol (4OP)

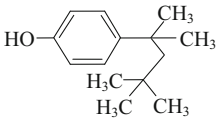


Fig. 7.5 4-*tert*-Octylphenol (4tOP)

Table 7.2 Concentration of xenoestrogens found in surface waters [39]

Country	4-*tert*-Octylphenol (mg/m^3)	4-Nonylphenol (mg/m^3)
Denmark	<0.1	<0.1–0.29
Germany	0.0004–0.036	0.001–0.221
	<0.01–0.189	<0.01–0.485
Holland	0.05–6.3	<0.11–4.1
England	<1	<0.2–180
Wales	<0.01–13	<0.03–5.2
USA	–	<0.11–0.64

It has been proven that nonylphenol causes endocrine disruption in fish. Nonylphenol, as well as other substances, interferes with endocrine activity (or has estrogenic activity), tending to bioaccumulate in the fatty tissues of living organisms. These compounds enter surface waters and groundwater with atmospheric wet (e.g., a rain) and dry (atmospheric aerosol particles) precipitation derived from landfill leachate and wastewater resulting from industrial agriculture. The value of the bioaccumulation concentration factor (BCF, the ratio of the pollutant concentration in an organism to that in the surrounding water) for nonylphenol is about 300 [40].

7.3.5 Metalloestrogens

Research carried out in recent years suggests that some metals and their compounds represent a novel class of compounds that interfere with oestrogenic activity (EDCs). So far, cadmium, copper, cobalt, nickel, lead, mercury, tin, chromium, and vanadium anions and arsenates have been identified as metalloestrogens, capable of binding to estrogen receptors. These metals are a part of a group of

elements referred to as "trace elements" and are present in very small (trace) amounts in both the Earth's crust and biological environments. As commonly understood, particularly in relation to environmental concerns, some of these metals are identified by the term "heavy metals" [41]. Large differences in the chemical properties of the individual elements of this group prevent definition of clear characteristics. Depending on the concentration and properties, they can adversely affect living organisms and also constitute an essential component in their construction and development. It is estimated that approximately 18 of these compounds are necessary for proper functioning of the human body.

Among the elements characterized by the highest ratios of accumulation (10–600) in the environment, the following are most frequently identified: cadmium, lead, zinc, and copper. Mercury and chromium are rarely identified [42]. All these metals pose a particularly high danger to humans if they exceed the levels of the biological barriers of living organisms at the lower levels of the food chain [43].

Metals classified as metalloestrogens can be found mainly in water and soil (Cr, Hg, Cu 1–800 μg/L and 40–459 mg/L). These metals are also found in fish (Ni, Cr, Hg, Pb, Cu 81–328 mg/L) and cereals (Cu 1–14 g/L), in air and cigarette smoke (Cd in the kidney of nonsmokers 15–20 mg/L and smokers 30–40 mg/L).

As already mentioned, some metals represent a new class of compounds that interfere with estrogenic activity. Estrogen receptors are proteins containing a DNA-binding domain that binds a metal, zinc. Interaction of zinc on the estrogen receptor with cysteine residues of DNA leads to the formation of so-called zinc fingers. Previous studies show that this metal can be replaced by another metal (e.g., nickel, copper) that blocks the DNA-binding domain and 17β-estradiol binding [44].

The ability of certain metals to bind to estrogen receptor α suggests that these metals can increase the risk of disorders of the endocrine system (hormonal system, endocrine). Long exposure of women to lead and mercury can lead, among other things, to infertility, miscarriage, and premature delivery.

According to recent studies, metals such as Ni, Co, Hg, Pb, and Cr^{6+} can cause breast cancer development and affect the kidney, lung, liver, and pancreas [42]. Arsenic also initiates the process of carcinogenesis (changes in body cells leading to tumor), induces sister chromatid exchange (exchange of genetic material between copies of the chromosome resulting from self-duplication), chromosomal conversion, and gene amplification, leading to different types of mutations. Arsenic also modifies the DNA repairing enzymes and interferes with replication of double-stranded DNA as a result of AS^{3+} interaction with thiol groups [45].

As already mentioned, metals that can cause disruptions in the endocrine system include cadmium, lead, mercury, cobalt, copper, nickel, tin, chromium, and anions of vanadium and arsenic [45, 46].

7.3.6 *Benzophenones*

UV filters are another group of organic compounds present in the aquatic environment. They are emerging contaminants and are causing legitimate concern. Every year, there are new formulations on the consumer market that are designed to provide reasonable protection of the skin against UV radiation. The first measurements of UV filters in seawater were started after noting that the mass extinction of coral reefs along the Australian coast correlated with crowded touristic places. Similar pollution problems are not confined to the seas and oceans, but also concern most inland waters. Streams, rivers, irrigation canals, lakes, and artificial lakes are becoming more and more exposed.

The best known and most commonly used ingredients of UV filters are compounds from the group of benzophenones (BPs), mainly 2,4-dihydroxybenzophenone (BP-1) and 2-hydroxy,4-methoxy benzophenone (BP-3). Their structures are depicted in Figs. 7.6 and 7.7.

These compounds act as chemical filters to protect against the adverse effects of UV radiation. This radiation can contribute to the destruction of collagen fibers, which in turn can lead to a reduction in immune response. Furthermore, UV radiation is also responsible for the formation of free radicals, which cause damage to the structure of proteins and can lead to tumor formation [47]. According to the published data, these compounds are present not only in wastewater, but also in marine waters, lakes, rivers, sewage sludge, and soil [48, 49].

In recent years, there have been a number of studies of the estrogenic properties of UV filters [50–52]. The results clearly showed that UV filters, even at low concentrations, can interfere with the endocrine system of mollusks, and in higher concentrations can be toxic to many aquatic organisms [53]. Research conducted on fish species (zebra fish) showed that fixed-dose UV filters (e.g., ethylhexyl-methoxycinnamate, abbreviated EHMC), even at low concentrations (2.2 mg/dm^3), can cause significant changes at the genetic level. The analysis showed that EHMC affects the transcription of genes involved in the metabolism of hormones in the body.

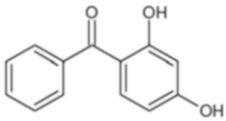


Fig. 7.6 2,4-Dihydroxybenzophenone (BP-1)

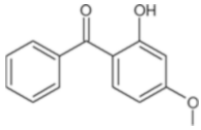


Fig. 7.7 2-Hydroxy,4-methoxy benzophenone (BP-3)

UV filters also negatively affect the processes of construction and reconstruction of tissues, the efficiency of the immune system, inflammation, and DNA damage [54].

The negative impact of UV filters on the endocrine system has been demonstrated in living organisms. Rats developed uterine hyperplasia and the fish *Pimephales promelas* showed an 800-fold increase in levels of the glycoprotein vitellogenin, an egg yolk precursor protein. However, the concentrations of UV filter used in the experiments were always higher than those reported in environmental samples [53–57]. It could therefore be concluded that these substances should not pose any threat to living organisms. However, one should not underestimate the impact of UV filter compounds on the environment. One should consider the risks associated with the lipophilic properties of UV filters, which can therefore be accumulated in the fatty tissues of living organisms, especially long-lived species. The highly lipophilic properties of BPs enable them to rapidly cross dermal tissue, which can cause bioaccumulation in the human body. A few hours after application, these BP UV filters can be detected in the plasma, bile, and urine [58]. Furthermore, some UV filters have been detected in human milk [59].

One should not ignore the possibility of mutual reinforcement of these compounds as a result of the presence of some other substances showing potential synergy properties [47]. Moreover, an important aspect of these compounds is their chemical transformation as a result of UV radiation to metabolites that are have potentially comparable or even stronger adverse effects on the environment [60].

The above-mentioned research activities show that the concentration of these compounds depends on the location and time of sampling. In addition, the season and even the day of sampling are important. During the summer, especially on sunny days, a significant increase can be seen in the concentration of diphenylketone derivatives in waters, with the highest values in the afternoon. Table 7.3 summarizes reported concentrations of the three most commonly used BPs in environmental samples, and Table 7.4 shows the concentrations of UV filters in the environment and biota [58].

BPs are used not only in specialized cosmetic preparations for skin protection against the carcinogenic effect of UV radiation; they are also added to pharmaceuticals and many everyday products such as body lotions, shampoos, bubble baths, and hair sprays. These compounds are used for the packaging of synthetic materials in order to extend the shelf life of stored products that could be affected by the influence of sunlight. Other application areas are tire additives, castings, coatings, pigments, textiles, and other products, to increase their resistance to UV radiation [49, 64, 73, 74].

Undoubtedly, because of their ability to absorb harmful UV-B radiation, BPs are the most commonly used component of UV filters all over the world. However, the European Union requires systematic monitoring of their content in cosmetics because of their potential to cause skin allergies and the fact that BP-3 and its metabolite BP-1 have suspected estrogenic activity [49].

The literature data suggest that BPs are generally efficiently removed from wastewater by biological processes [48, 61]. In various sewage treatment plants, removal efficiency of BP-3 ranged from 28–30 % to 68–96 %. The authors point out that a lack of biological wastewater treatment clearly reduces the effectiveness of their removal in treatment plants [61].

Table 7.3 Concentration of benzophenones in environmental samples

Compound	Sample type	Period of sampling	Concentration ($\mu g/m^3$)	References
Benzophenone-4	Raw sewage	Summer	1481	[61]
	River water		849	
	Sea water		138	
Benzophenone-3	Raw sewage	Summer	1195	[62]
Benzophenone-4			4150	
Benzophenone-3	Lake water	Cold	35	[48]
		Hot	125	
Benzophenone-3	Industrial wastewater	Higher concentrations in summer, lower concentrations in other seasons	6–697	[63]
	Domestic sewage	Higher concentrations in summer, lower concentrations in other seasons	720–7800	
Benzophenone-3	River water	Spring	300	[64]
Benzophenone-1			1000	
Summary of B3 and B1	Raw sewage		2700–4800	

Table 7.4 Concentration of UV filters in the environment and biota [55]

Environmental sample	UV filter	Maximum concentration (ng/L, mg/kg dw)	Location	Reference
Lake water	4-MBC BP-3 EHMC OC BM-DBM	80 125 19 27 24	Switzerland	[52]
	BP-3 EHMC Et-PABA OC	85 92 34 31	Slovenia	[65]
	BH	85	Korea	[66]
River water	HMS BP-3 EHMC OC	345 114 88 34	Slovenia	[65]
	DHB	47	Korea	[66]

(continued)

Table 7.4 (continued)

Environmental sample	UV filter	Maximum concentration (ng/L, mg/kg dw)	Location	Reference
Seawater (beach)	4-MBC BP-3 EHMC OC	488 269 238 4461	Norway	[67]
Raw drinking water	EHMC	5610	California	[68]
Raw wastewater	4-MBC BP-3 EHMC OC	6500 7800 19,000 12,000	Switzerland	[69]
	BP-3 EHMC	6240 400	California	[68]
Treated wastewater	4-MBC BP-3 EHMC OC	2700 700 100 270	Switzerland	[69]
Swimming pool water	4-MBC BP-3	330 400	Slovenia	[65]
Fish (lakes)	4-MBC HMS EHMC BP-3	3.80 mg/kg (lw) 3.10 mg/kg (lw) 0.31 mg/kg (lw) 0.30 mg/kg (lw)	Germany	[70]
	4-MBC BP-3 EHMC OC	0.17 mg/kg (lw) 0.12 mg/kg (lw) 0.07 mg/kg (lw) 0.02 mg/kg (lw)	Switzerland	[69]
Fish (rivers)	4-MBC OC	0.42 mg/kg (lw)[a] 0.63 mg/kg (lw)[a]	Switzerland	[71]
Sewage sludge	4-MBC EHMC OC OMC	1.78 mg/kg (dm)[a] 0.11 mg/kg (dm)[a] 4.84 mg/kg (dm)[a] 5.51 mg/kg (dm)[a]	Switzerland	[72]

dw dry weight, *lw* lipid weight, *dm* dry matter, *EHMC* ethylhexyl-methoxycinnamate, *4-MBC* 3´-(4´-methylbenzylidene)camphor, *OCR* 4-octocrylene, *BP-3* benzophenone-3, *OC* octocrylene, *Et-PABA* 2-ethylhexyl 4-dimethylaminobenzoate, *BM-DBM* butyl methoxydibenzoylmethane, *HMS* 3,3,5-trimethylcyclohexyl salicylate, *BH* benzhydrol, *DHB* 2,4-dihydroxybenzophenone, *OMC* octyl 4-methoxycinnamate
[a]Mean concentration

7.4 Nitrosamines

Nitrosoamines (Fig. 7.8) are the most dangerous EDCs, showing several mutagenic and carcinogenic properties [75].

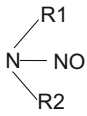


Fig. 7.8 General formula for nitrosamines

N-Nitrosamines, according to the directive of the Polish Ministry of Health and Social Security, as well as EU directive 93/11/EEC [76, 77], are rated as carcinogenic or probably carcinogenic compounds [78].

Every year, over 50 million tons of waste containing nitro-related compounds is created all over the world. Nitrocompounds are formed by nitration of not only amines, but also amides, urea, guanidines, carbamates, cyanides, and sulfonamides. Because of their chemical properties they are divided easily into hydrolyzable *N*-nitrosamides and relatively stable *N*-nitrosamines. Nitrosamines are stable compounds that slowly decompose when exposed to light or in acidic aqueous solutions.

Large amounts of nitrosamines leak into the environment from the pharmaceutical and food industries, plastics industry, textile industry, waste transport (motor vehicles), industrial effluents (dyes, lubricants, rubber), and the production of solvents. Fuel manufacturing plants and oil refineries are also important emitters of nitrosamines, as well as landfills and fossil fuel combustion processes (to produce heat and power). These compounds naturally penetrate the environment through animal droppings.

Nitrosamines are formed from secondary and tertiary amines under the influence of various kinds of microorganisms, and at elevated temperature. Risk of their formation increases during technological processes. They are formed during bad food storage or in the gastrointestinal tract under the influence of gastric juice. The most common nitrosamines are the following:

- Nitrosodimethylamine (NDMA), arising from glycine and valine; in beer its precursor can be sarcosine (*N*-methylglycine)
- Nitrosodiethylamine (NDEA)
- Nitrosomethylethylo-amine (NMEA), resulting from alanine
- Nitrosodipropylamine (DPNA)
- Nitrosopiperidine (NPIP)
- Nitrosopyrolidyne (NPYP), arising from proline

N-Nitroso compounds differ in their biological activity. In contrast to the *N*-nitrosoamides, *N*-nitrosamines show mutagenic and teratogenic effects. *N*-Nitroso compounds are rapidly absorbed from the gastrointestinal tract and their biological half-life is less than 24 h. As a result of experimental studies involving 300 *N*-nitroso compounds, it was found that more than 80 % are carcinogenic. *N*-Nitrosodimethylamine (NDMA) at a concentration of 20 ppm alkylated DNA chains, causing liver cancer.

N-Nitroso compounds were detected in low concentrations in air, water, and food, particularly meat products to which nitrate(III) had been added and certain fish products. NDMA was detected in urban air samples. The presence of *N*-nitroso

compounds has also been found in purified water (water supply) and rivers. In experimental studies, the formation of *N*-nitrosamines was demonstrated in living organisms through reaction of nitrates (III) and (V) in the presence of amines and amides, as well as the quaternary ammonium bases present in foods.

The formation of *N*-nitrosamines is supported by carbamate pesticide residues and certain medications. Certain compounds (chlorides, iodides, bromides, tricyanides) catalyze the formation of *N*-nitroso compounds from secondary amines and nitrates(III); others inhibit this process (ascorbic acid, gallic acid, sodium sulfite). *N*-Nitrosamines can be formed at the time of processing and storage of food, as well as in the gastrointestinal tract of animals, in the environment of gastric juice. Numerous works on nitration showed that these reactions depend not only on the presence of precursors, but also on their quantity, temperature, and pH. With weaker basic amines, the reaction is faster, with increased productivity. However, nitrosamines formed with strongly basic amines (dimethyl and diethyl) are present in larger quantities. The formation of *N*-nitrosamines in the gastrointestinal tract of humans and animals is also correlated with the presence of various other non-aminated food ingredients. Phenolic compounds are also nitrated. Such reactions are catalyzed by, for example, thiocyanates, which are components of saliva. For cigarette smokers, the reactants found are nicotine and nitrogen oxides.

There is considerable evidence that the processes related to food processing on both industrial and household scales influence the formation of nitrosamines. For example, in fresh fish the content of *N*-nitrosamines is greater than 5 g/kg, whereas in processed fish (stored) it reaches 13 g/kg. The amount of these compounds is higher in smoked products. Studies of 300 types of processed pork showed that the average content of NDMA is 3 g/kg, but the highest concentration was as high as 50 g/kg.

Cheese is another food product in which some carcinogenic nitrosamines are created under the influence of heat. Amines are present in cheese as a result of the degradation of amino acids, which are the basic building blocks of proteins. Combining such products as ham and cheese can cause excessive concentrations of *N*-nitrosonornicotine (NNA) in the body. Also, high temperature during treatment of these products (e.g., preparation of pizza or casseroles) can cause the dose limit to be exceeded. There are no exact data on the formation of nitrosamines in cheese, although nitrates (III) and (V) are widely used for the production of some types. It is supposed that nitrosamines in preserved meat could be formed as a result of the interaction between nitrate(III) and spices such as black pepper and paprika.

Harmful levels of nitrosamines were also detected in beer, which forced the producers to change the technology of the production process. In beer, high levels of nitrosamines were detected that reached 3 mg/L. In Spain, as many as 52 % of the products were contaminated with nitrosamines, mainly NDMA and *N*-nitrosodiethylamine (NDEA). These compounds are formed by direct drying of barley malt in an oven. Initiation of the reaction of nitrogen oxides is provoked by the so-called air process, with an air temperature in the oven of 1500–1800 °C. Changing the beer production process by introduction of a heat exchanger reduced air temperature to 100 °C. This improvement significantly decreased the amount of

nitrosamines produced. Their concentration in different kinds of beer was similar and depended primarily on the amount of added malted barley. Beer type also influences amount of NNA detected. Alcoholic beer contains less harmful compounds than soft drinks, and light beer is less harmful than dark beer. The presence of NNA was also detected in some other alcoholic beverages (brandy, rum) and drugs (aminophenazone, oxytetracycline).

The most important sources of *N*-nitrosamines in terms of NDMA in the human diet are the following: fried bacon, smoked meat, smoked fish (0.4–440 mg/kg), wine (10–21 mg/kg), and beer (0.1–0.5 mg/kg) [79].

The presence of *N*-nitrosamines in the atmosphere, workplace, food, and articles of common use should be monitored and later eliminated. Checking the presence of nitrosamines is particularly important for products intended for direct contact with food and the human body. This applies especially to rubber articles intended for infants and children (e.g., pacifiers and toys) and products used in the food industry (e.g., hoses, belts, machine parts). Despite the fact that nitrosamines in rubber products are present in very low concentrations (parts per million and lower), these compounds can threaten health.

N-Nitrosamines in rubber products are by-products of the reactions taking place during the vulcanization of rubber mixtures [80]. They are formed from some chemical compounds such as secondary amines (accelerators, antioxidants) via nitrosation by nitrogen oxides present in the surrounding air. These reactions occur inside the rubber product and on its surface. The resulting *N*-nitrosamines from the interior of the rubber can diffuse to the surface of the article, and then to the environment or media in which the product is used. *N*-Nitrosamines can also be incorporated in the rubber mixture by contamination of raw materials during preparation.

7.4.1 Determination of Nitrosoamines

Based on animal studies, *N*-nitrosamines are compounds with proven carcinogenic effect. The search for sources and reduction of human exposure to their action is now one of the most important research problems. Currently, a major problem is the presence of *N*-nitrosamines and their precursors in food. Determination of *N*-nitrosamines in food relies on isolating these compounds by vacuum distillation in a basic medium, in the presence of liquid paraffin, followed by extraction with dichloromethane, pre-concentration of the sample, and analysis using GC. A thermal energy analyzer coupled to a gas chromatograph can serve as an efficient detector for these compounds.

Intensive nitrogen fertilization with mineral compounds supports the formation of nitrosamines in water. Nitrogen fertilizers are washed away with the rain from grass fields and thus enter surface waters such as lakes or rivers. These compounds also accumulate in soil, even for a period of 200 days. At pH 7–8, the risk of creation of these compounds is smallest. Above pH 10, the formation of

nitrosamines dramatically increases. NNA are also formed during chlorination of water through the decomposition of other harmful substances.

For pharmaceutical purposes, it is necessary to determine *N*-nitrosodiethanolamine in trolamine. This compound is characterized by high volatility and low concentration in real samples. Thus, to determine trace amounts of this compound, preconcentration is needed. Vacuum distillation is one of the possible methods [81]. To quantitatively determine nitrosamines, GC and HPLC are often applied [82, 83]. According to the fifth European Pharmacopoeia, the maximum concentration of *N*-nitrosodiethanolamine should not exceed 25 ppb.

7.5 Flame Retardants

Flame retardants (FRs) are a group of chemicals, organic and inorganic, that are added to materials to reduce their flammability and reduce the spread of fire after ignition.

Flame retardancy can be accomplished by:

- Changing the pyrolysis process by reducing the amount of flammable, volatile low molecular weight substances
- Speeding up the process of breaking the polymer chain
- Creating a less combustible, charred-shell barrier between the polymer and the heat source
- Cutting off the air supply
- Limiting the spread of fire by deactivating free radicals
- Reducing the amount of heat emitted during smoking through endothermic reactions such as dehydration and decarboxylation [84, 85]

Use of FRs results in limited combustibility of substances, reduced degree of spread of fire, and even the ability to avoid fire. Flame retardants also contribute to prolongation of the time needed to exit rooms and buildings during a fire and increase the probability of extinguishing the fire when the fire brigade arrives.

Flame retardants can be divided as follows [86]:

- Inorganic compounds, such as aluminum hydroxide and magnesium hydroxide
- Halogenated derivatives of organic compounds, such as chlorinated and brominated flame retardants (BFRs)
- Organophosphorus compounds (OPFRs)
- Other compounds, such as melamine, melamine cyanurate, and red phosphorus

Halogen derivatives of organic compounds (mainly substances containing chlorine and bromine) can be used as FRs. These compounds are highly effective and are among the most efficient FRs in terms of economic aspects. However, these compounds are highly toxic for humans and for the environment and involve use of Sb_2O_3. Brominated FRs are generally twice as effective as the chlorinated compounds and have a higher decomposition temperature. The products of combustion

of these materials are often acidic, and therefore they are not used in the production of cables. The action of this group of FRs is to remove energetic free radicals and hydroxyl hydrogen formed during combustion [87–89].

The most important OPFRs are phosphinates, phosphonates, and phosphoric esters (V). The proportion of phosphorus in OPFRs has an effect on their ability to delay burning of a material. Thus, compounds in which the substituents are aliphatic chains are more desirable than aromatic compounds, because the share of phosphorus in aliphatic substituents is larger. A limitation in the use of aliphatic compounds is their greater susceptibility to hydrolysis compared with compounds having aromatic substituents. In order to increase the proportion of phosphorus and reduced susceptibility to hydrolysis, attempts have been made to synthesize compounds containing both aromatic and cyclic substituents. Further increase in the flame retardant properties of OPFRs can be achieved by the addition of chlorine, bromine, or nitrogen atoms to their structure. Halogen atoms are added mainly to an alkyl chain, and nitrogen atoms are mainly added as an amino group. Strengthening of flame retardancy is caused by a synergistic effect between phosphorus and the introduced chlorine, bromine, and nitrogen [84, 90, 91].

Retardants are mainly used as additives for plastics, particularly those used in the manufacture of electrical and electronic equipment. FRs are added to circuit boards, cables, connectors, plugs, and house component devices. These compounds are used in the production of engineering plastics, thermoplastic and elastomeric elements, and insulation. Flame retardants can also be found in furniture, mattresses, carpets, curtains, clothing (mainly protective or sweatshirts for children), and polymeric materials used for the production of cars, buses, airplanes, and military equipment. FRs have been used wherever material must meet safety standards regarding flammability [87].

The most frequently used BFRs are polybrominated diphenylethers (PBDEs), tetrabromobisphenol A (TBBPA), and hexabromocyclododecane (HBCD). The use of FRs has been growing rapidly in recent years. BFRs are the most often used FRs, and their market is still growing. However, the estimated annual use of OPFRs in Western Europe was almost twice that of all BFRs combined. Many FRs have been banned for use because of their potential toxicity, environmental occurrence, and accumulation in human tissue. FRs taken off the market are likely to be replaced by others. Although the REACH (registration, evaluation, authorization and restriction of chemicals) regulatory system has been introduced in Europe to improve protection of human health and the environment, it is still necessary to monitor FRs in environmental samples [84, 88].

Despite the use of FRs on a large scale in different industries, in many countries their use is being systematically reduced. This is directly connected with the high toxicity of these compounds (particularly halogen derivatives of organic compounds). FRs used in the textile industry can cause irritation of the skin and mucous membranes. FRs, like other compounds during combustion, produce a number of toxic products (CO, CO_2, PO_x, NO_x, HBr, HCl, and HCN), which can cause allergies, respiratory allergies, or severe intoxication. A very dangerous feature of FRs is their chronic toxicity, associated with the formation of dioxins during the

decomposition of halogen FRs. Many organophosphorus compounds exhibit neurotoxic effects, and some show carcinogenic and mutagenic effects [84, 88].

Proven durability, bioaccumulation, and toxicity to the environment and living organisms has led to increased control over many halogenated FRs, and even to a total ban on the production of some of them. These limitations have led to increased interest in organophosphorus agents and an increase in their importance and overall use, which in the European Union in 2006 was 20 % of total usage [84, 92]. The primary source of FRs in the air is their emission from paints, electrical equipment, construction and finishing materials, furniture, household resources, and others. Because those compounds are designed not to lose their properties over many years they are a stable component of the materials in which they are contained. The disadvantage of this stability is the fact that, even after using the object or material, FRs can penetrate into the environment along with rainfall, municipal and industrial sewage, and by many other productive activities and daily human activity [88, 92]. Prolonged exposure to FRs, which bioaccumulate, can cause adverse health effects. These compounds can mimic the action of natural hormones, have an impact on their metabolism, or can activate or block the action of receptors or even change their amount. The most common effects of exposure to FRs are weakening of the body's defense system, reduction in fertility, neurobehavioral developmental defects, and cancer. In addition, FRs can accumulate in adipose tissue, blood, and in the body. Children who are exposed to FRs during fetal development can have growing disorders, mental or physical retardation, or impaired development of the skeletal, nervous, and endocrine systems [84, 88, 92].

Therefore, FRs are determined in different matrices, both environmental and biological. The existing literature mainly focuses on the determination of compounds that have been available for many years, or even long after their production was banned (e.g., PCBs). The desirability of such research is justified by the fact that many of these compounds are still present in real samples as a result of their high durability and release to the environment from products produced before the introduction of prohibitions (and still present on the market). That is why it is important that, along with development of new FRs, analytical procedures are provided for their determination in various matrices, especially those with which we are in most frequent contact. The literature describes FR determination mostly in samples of surface water [93, 94], soils [95, 96], sediments [97, 98], and dust [99, 100]. These environmental matrices are in often contact with materials that contain FRs. Moreover, from these matrices it is very simple for FRs to come into contact with animals and humans; ttherefore, it is very important to monitor the concentrations of FRs in those matrices.

Because the environmental matrix is very rich in various kinds of interferents and the FR concentration is relatively low, there is a need to use various kinds of extraction techniques to separate these compounds from environmental samples and to enrich and purify the samples. Depending on the type of matrix, different techniques can be applied. For water samples, first a pre-cleanup step is needed and then the most common technique used is solid phase extraction (SPE) with different SPE columns (C_{18}, ENV, HLB, PAD3) and different eluents. To enrich the FRs

from water samples, volumes of 500 mL and even 1000 mL are used [93, 94, 101]. The most important finding from these studies is the fact that some of the compounds are found in surface water in quite high amounts. Tris(2-chloro-1-methylethyl) phosphate (TCPP) was quantified at very high levels (26 μg/L) [94]; however, most of these compounds were found at the nanogram per liter level and did not exceed 500 ng/L.

Soil, sediment, and dust samples were prepared in a similar way before analysis. After the pre-cleanup steps and homogenization, FRs were extracted from samples using different solid–liquid extraction techniques. The most commonly used technique was accelerated solvent extraction (ASE), which enables the fast extraction of samples using different solvents such as hexane and dichloromethane [98–100]. Other commonly used techniques for these samples were ultrawave-assisted extraction (UAE) [97], which also enabled quick extraction, and the more time-consuming but very efficient technique, Soxhlet extraction [96]. Some authors have also described less common techniques such as microSPE [95]. There is also information that many FRs that are no longer produced (mainly PCBs and PBDEs) are present in dusts, soils, and sediments in very high amounts, even 390 μg/g [98].

The widespread presence of FRs in environmental samples can be a big threat to animals and fish, and also directly to humans. This is why authors have described the determination of selected FRs in fish and bird tissues [102] and also in human blood and serum [103]. In these works, the authors prepared and optimized methods for use when such threats occur. For animal tissues, microwave-assisted extraction was proposed for the preconcentration of FRs. For human serum and blood, SPE was proposed.

The final concentration of examined FRs can be determined using different techniques. Extracts prepared using techniques mentioned earlier were mostly analyzed using chromatographic techniques, especially for determining the concentration of various organic compounds. In the literature presented in this chapter, the most commonly used techniques were GC coupled with MS [9, 93, 96, 98, 102] or with tandem MS/MS [98, 100, 103]. One author has described the use of different detectors combined with a GC system (μECD) [95]. A few researchers used liquid chromatography. In one case, HPLC coupled with tandem MS/MS was used [99] and in others ultra-HPLC (UHPLC) was combined with a tandem MS/MS detector [100] or a UV detector [101]. The use of UHPLC can shorten the analysis time by up to a factor of ten.

7.6 Summary

This chapter presents basic issues related to the trace analysis of selected compounds. Particularly important is the detection and identification of carcinogens and estrogenic compounds, being a main cause of cancer. For this reason, attention is focused on selected organic compounds having such properties. These include such

carcinogens as benzophenones, *N*-nitrosamines, estrogen-active compounds, phthalates, and nonylphenols. In particular, bisphenol A and its derivatives show estrogenic properties, which recently drew the attention of researchers. In addition, this chapter discusses some issues related to the analysis of flame retardants, because of their common use and the widespread presence of these compounds in the natural environment.

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Chapter 8
Analysis of Drug Impurities

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8.1 Introduction

Although the development of trace analysis is considered to have begun with studies related to the determination of trace elements, from the pharmaceutical point of view, the beginning of this field of science should be sought in the analysis of drugs and especially in the search for active compounds in different types of pharmaceutical decoctions, tinctures, and macerations. Isolation of opium and later morphine from poppy juice, or isolation of quinine from cinchona tree bark, were nothing other than preparation and concentration of a sample before performing trace analysis [1–3].

The aim of this chapter is to discuss one of the most important problems in the trace analysis of pharmaceuticals: analysis of drug impurities. The term "drug impurity" is exchangeable with the term "drug purity," and usually means chemical, chromatographic, or pharmacopoeial purity. According to the United States Pharmacopeia [4] and European Pharmacopeia [5], every drug authorized for marketing in the USA and EU countries has to be of appropriate purity, including both chemical and microbiological purity. A drug that meets the pharmacopoeial standards can contain only chemicals and microorganisms in amounts allowed by the pharmacopeia.

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Before discussing investigation of the chemical purity of drugs and trace analysis of chemical impurities in medications, a definition of impurity should be given. The most current definition states "an impurity of a drug is every chemical compound different in molecule and composition from the active substance of the medication, excluding water, which is not considered as an impurity" [5].

According to this definition, impurities also include all excipients used to form the drug formulation, giving it adequate consistency, stability, solubility, taste, and other factors. This brings us to an apparent contradiction with the existing definition of chemical impurity; in pharmaceutical analysis and analysis of drugs, all excipients are treated the same as the active substance. This means that they have to meet the same standards in terms of their purity, cannot contain impurities in quantities larger than that of the active substance, and have to meet other standards referring to the chemical structure, properties, and so on.

In practice, it is impossible to obtain medication of 100 % purity, which makes it necessary to check the impurity profile of the drug. Investigations involve the isolation, identification, and quantification of the impurities, and in many cases define their biological activity and toxicity. Performing such studies is an important part of the development of a drug and is a part of safe pharmacotherapy. The goal is to prevent tragedies, such as one in 1938 when 105 people died after administration of "Elixir Sulfanilamide" that was contaminated with diethyl glycol [6].

Determination of the contamination profile of the active substance (active pharmaceutical ingredient) and the final product (drug product) is a broad and time-consuming process, the goal of which is to guarantee the quality of the medicinal product. Quality of the product is one of the most important factors in terms of the safety of pharmacotherapy.

Following the ICH (International Conference on Harmonization of Technical Requirements of Pharmaceuticals for Human Use) definition, an impurity is every separate chemical particle present in the active substance and final pharmaceutical product that is not the active substance or excipient. This includes all optical isomers, products of degradation, and polymorphic forms. The physicochemical properties and biological activity of drug impurities can be similar or different to those of the active substance. Therefore, they can influence the human body in the same way as the medicinal product or differently [7] and lead to an increase or decrease in drug activity.

8.2 Regulations for Control of Drug Impurities

Pharmacopoeial monographs on medicinal substances describe basic requirements that need to be met to assure appropriate quality of the product. Most of the monographs indicate purity at the 98–99 % level, which means that a content of 1–2 % impurities is allowed. In cases of medications used in high doses, such as vitamins or antibiotics, which have daily doses from few hundred milligrams up to a few grams, a 1–2 % level of impurities can have serious consequences for patients.

More restrictive indications regarding purity of medicinal products are presented in ICH documentation Q3A, Q3B, and Q3C [7–9]:

– Q3A: This document describes impurities in pharmaceutical materials. Based on this regulation, the following levels of impurities must be evaluated for each new medicinal substance:

  • Organic impurities: all impurities with known or unknown structure if they are present in concentrations >0.1 %; all impurities of harmful properties if they are present in concentrations <0.1 %; total quantity of impurities
  • Inorganic impurities
  • Levels of solvent residues

  Q3A regulations do not cover substances of biological or biotechnological origin, proteins, and radiopharmaceuticals [7].
– Q3B: This document also describes impurities in pharmaceutical preparations. The regulations focus mainly on the levels of products of degradation and products of reactions of the active substance with excipients or components of the packaging. The document also specifies the levels at which the impurities have to be identified and properly qualified. Based on these regulations, pharmaceutical products are divided into groups according to total daily dose of the active substance: <1 mg, 1–10 mg, 10–100 mg, 100 mg to 2 g, and >2 g. For example, medications for which the daily dose is within 1–10 mg and their quantity is higher than 0.5 %, the ICH guidelines require that the structure and biological activity of impurities are determined. If the level of impurities is lower than 0.5 %, then the daily amount received by the patient with the medication has to be calculated, and if it is higher than 20 μg/dose, the structure of the impurity has to be established. If the amount is higher than 50 μg/dose, then the biological activity of the impurity has to be determined (Table 8.1) [8].
– Q3C: This document focuses on the residues of solvents in medications. It describes both organic and inorganic solvents used in the synthesis of both the active substance and the excipients. According to ICH guidelines, solvents can be divided into three classes:

  • Class I: solvents with proven or potential carcinogenic properties, and solvents hazardous to the environment, such as benzene, 1,2-dichloroethane, 1,1-dichloroethane, and carbon tetrachloride. These solvents should not be used in the pharmaceutical industry. If they have to be used, then their levels in pharmaceutical products must be monitored, and they cannot exceed ICH guidelines (Table 8.2) [9].
  • Class II: solvents with possible toxic activity or that present reversible toxicity. This class includes 25 solvents, such as acetonitrile, methanol, cyclohexane, and methylene chloride. For each solvent there is a permitted daily exposure dose (PDE) and an acceptable level of concentration in the final product (Table 8.2).

Table 8.1 ICH guidelines for reporting, identification, and qualification thresholds for impurities in drug substances and drug products [7, 8]

	Threshold		
Maximum daily dose	Reporting [%]	Identification [%]	Qualification [%]
Drug substance			
≤2 g	0.05	0.10 or 1.0 mg per day	0.15 or 1.0 mg per day
>2 g	0.03	0.05	0.05
Drug product			
≤1 g	0.1		
>1 g	0.05		
<1 mg		1.0 or 5 μg per day	1.0 or 50 μg per day
1–10 mg		0.5 or 20 μg per day	1.0 or 50 μg per day
10–100 mg		0.2 or 2 mg per day	0.5 or 200 μg per day
100 mg–2 g		0.2 or 2 mg per day	0.2 or 2 mg per day
>2 g		0.1	0.1

- Class III: solvents of low toxicity in humans, such as acetone, ethanol, and ethylene ether. The daily acceptable level for this class is 50 mg, but in some cases it can be exceeded. This class contains 27 solvents (Table 8.2).

Solvent residues are impurities that are hard to completely remove during the technological process. This brings the necessity to determine their levels in the final product. According to ICH standards, residues of Class I have to be always identified and quantified. Similar procedures concern the residues of Class II and III if the concentration levels exceed acceptable norms [9].

8.3 Characteristics of Drug Impurities

Drug impurities, based on their chemistry, can be divided into organic and inorganic substances. According to their origin, they can be divided into the following classes:

1. Impurities originating from the synthesis processes of the active substance or excipients
2. Impurities originating from processes of degradation of the active substance or excipients
3. Accidental impurities

Table 8.2 Classes of organic solvents according ICH guidelines Q3C [9]

Class I		Class II		Class III	
Solvent	Concentration limit (ppm)	Solvent	Concentration limit (ppm)	Solvent	Concentration limit (ppm)
Benzene	2	2-Methoxyethanol	50	1-Butanol	0.5
Carbon tetrachloride	4	Methylbutyl ketone	50	1-Pentanol	0.5
1,2-Dichloroethane	5	Nitromethane	50	1-Propanol	0.5
1,1-Dichloroethene	8	Chloroform	60	2-Butanol	0.5
1,1,1-Trichloroethane	1500	1,1,2-Trichloroethene	80	2-Methyl-1-propanol	0.5
		1,2-Dimethoxyethane	100	2-Propanol	0.5
		Tetralin	100	3-Methyl-1-butanol	0.5
		2-Ethoxyethanol	160	Acetic acid	0.5
		Sulfolane	160	Acetone	0.5
		Pyridine	200	Anisole	0.5
		Formamide	220	Butyl acetate	0.5
		Hexane	290	Dimethyl sulfoxide	0.5
		Chlorobenzene	360	Ethyl acetate	0.5
		1,4-Dioxane	380	Ethyl ether	0.5
		Acetonitrile	410	Ethyl formate	0.5
		Dichloromethane	600	Formic acid	0.5
		Ethyleneglycol	620	Heptane	0.5
		Tetrahydrofuran	720	Isobutyl acetate	0.5
		N,*N*-Dimethylformamide	880	Isopropyl acetate	0.5
		Toluene	890	Methyl acetate	0.5
		N,*N*-Dimethylacetamide	1090	Methylethyl ketone	0.5

(continued)

Table 8.2 (continued)

Class I		Class II		Class III	
Solvent	Concentration limit (ppm)	Solvent	Concentration limit (ppm)	Solvent	Concentration limit (ppm)
		Methylcyclohexane	1180	Methylisobutyl ketone	0.5
		1,2-Dichloroethene	1870	Pentane	0.5
		Xylene	2170	Propyl acetate	0.5
		Methanol	3000	*tert*-Butylmethyl ether	0.5
		Cyclohexane	3880		
		N-Methylpyrrolidone	4840		

The largest group is *impurities originating from synthesis* of the active substance. According to Sandor Görög and coworkers [10–12, 14, 16] they can be divided into the following groups:

- Substrates and semi-products of the synthesis: predicted impurities (probable), the structure of which can be established using the synthesis process reactions
- Products of competitive reactions: predictable impurities originating from the reactions parallel to the main synthesis reaction
- Products of side reactions: impurities for which the structure is hard to predict
- Impurities of the final product originating from substrate impurities: impurities of the substrates can lead to impurities of the final product, as they undergo the same reactions, often resulting in isomeric impurities
- Impurities of the final product originating from the solvents: solvents and their impurities can be present in the final product of the reaction
- Impurities originating from the reaction catalysts: it is possible that catalysts used in the synthesis can become the substrate of the reaction, and therefore cause contamination of the final product
- Inorganic impurities: buffers, inorganic reagents, catalysts, heavy metals and their ions (mostly originating from the apparatus), and reduction and oxidation factors

A separate group of impurities are the *products of degradation*. These impurities can be identical to impurities originating from the synthesis process, or they can have a different structure. Products of degradation are formed in solution and in the solid phase, and they come mostly from the reactions of hydroxylation, oxidation, reduction, isomerization, and epoxidation. Products of degradation can have their own pharmacological activity, and sometimes they even show toxic effects. Stability testing of drugs can specify the mechanism and kinetics of drug degradation, determine the influence of different factors on drug stability, and specify what storage conditions minimize degradation of the product.

Another group of impurities are *accidental impurities* [13–15]. These are any chemical substances that are not connected in any way with the synthesis or degradation of the drug. This group includes:

- Metals: usually originating from the apparatus used for synthesis, currently reduced by using more advanced technologies and materials
- Disinfectants: used for preparation of the production lines
- Herbicides, pesticides, heavy metals: in drugs of plant origin

Organic impurities can be separated into *chiral* and *genotoxic impurities*. The chemical structure and physicochemical properties of drugs are strictly connected with their pharmacological activity, therefore small changes, such as a different configuration at the asymmetrical carbon, can change the biological activity of the molecule. For chiral drugs, optical isomers can be considered impurities, for example, the *S*-enantiomer of penicillamine is used in rheumatoid arthritis, but the *R*-enantiomer shows significant toxicity [13–15].

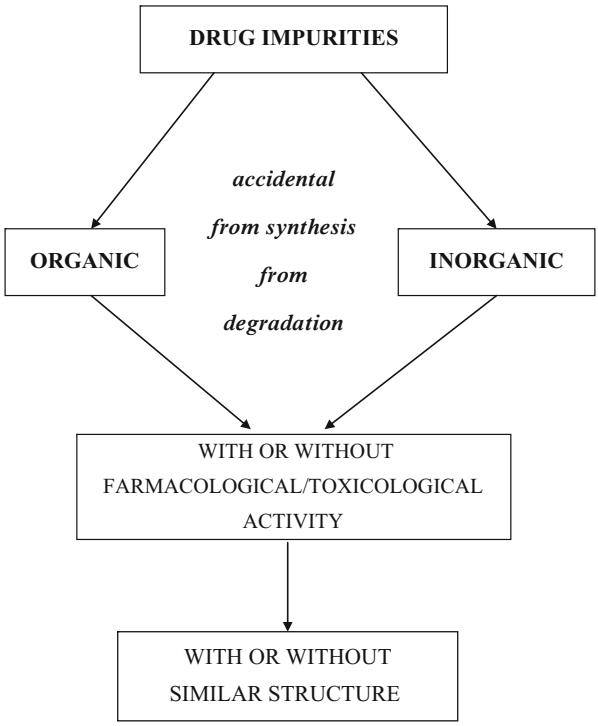

Fig. 8.1 Schematic categorization of drug impurities

Genotoxic impurities are a separate group of organic impurities as a result of their biological properties. Those compounds can cause genetic mutations, chromosomal changes, and induce cancer. Even small or trace amounts of these impurities in the final pharmaceutical preparation is strongly undesirable. This group includes such compounds as aromatic amines, epoxides, peroxides, and heterocyclic nitrogen compounds [17].

In summary, impurities in the active substance and final pharmaceutical preparation can show structural similarity to the drug molecule or present completely different chemical characteristics, can present no biological activity or show significant pharmacological activity (even toxicity), and can cause an increase in side effects (Fig. 8.1)

8.4 Instrumental Methods in Analysis of Drug Impurities

Establishing the impurity profile of an active substance or final pharmaceutical product requires use of several analytical methods to fully and precisely identify all of the impurities.

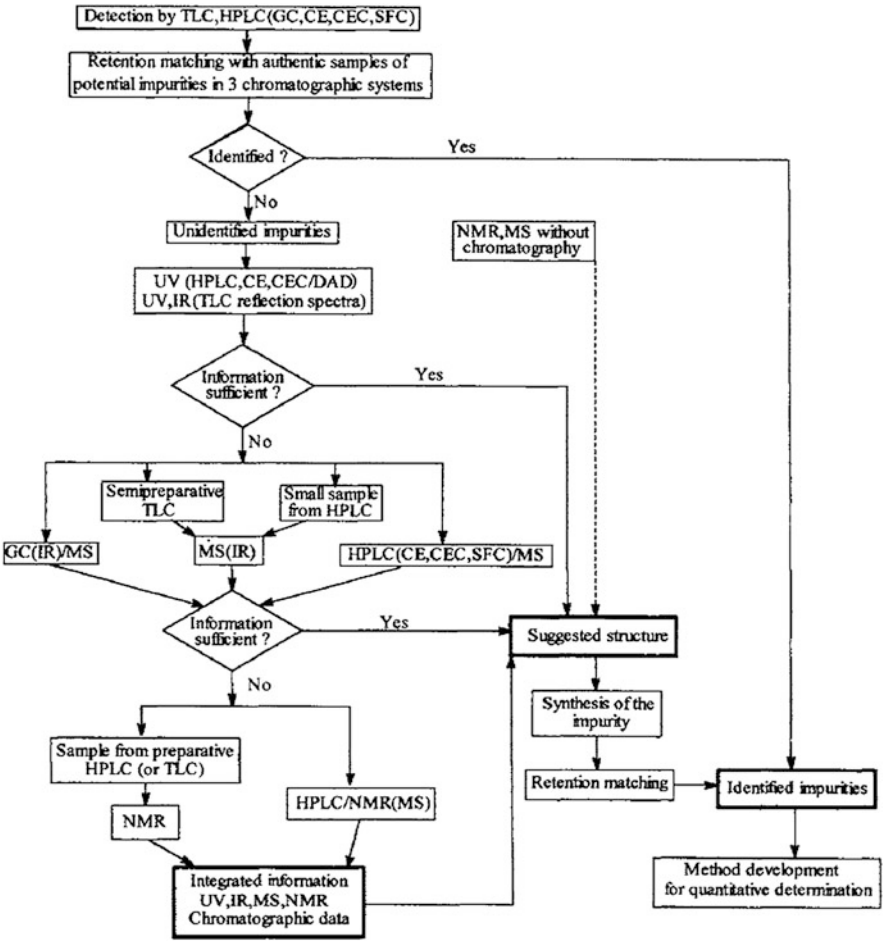

Fig. 8.2 General scheme for the detection, identification, and structural elucidation of related organic impurities in drugs [19]

Methods in the analysis of drug impurities (e.g., ultraviolet, UV; Fourier transform infrared, FT-IR; nuclear magnetic resonance, NMR; mass spectrometry, MS) are used to separate, identify, and quantify impurities, as well as establish their structure. Currently the most efficient methods seem to be combined techniques such as GC-MS, LC-MS, liquid chromatography–diode-array detection–mass spectrometry (LC-DAD-MS), LC-NMR, LC-DAD-NMR-MS, etc. [18–20].

Figure 8.2 shows the decision scheme for establishing the purity profile of an active substance. First, the impurities are separated using chromatographic or electrophoretic methods, in at least three distribution systems. The retention times are compared with the retention times of standards, and the eluents quantified. Next, spectroscopic methods (UV, FT-IR, MS) combined with chromatographic/electrophoretic methods are used to establish the structures of unknown impurities. If this

is not enough, then preparative liquid chromatography (thin layer chromatography, TLC; high-performance liquid chromatography, HPLC) should be performed, and the obtained results checked with the use of NMR spectroscopy or using a HPLC-NMR-MS combination. In the next stage, the identified impurities are synthesized, analyzed chromatographically, and the retention times of the synthesized standards compared with those of the impurities. Using this scheme to establish the impurity profile of a drug guarantees that the impurities are adequately indentified [19].

Based on collective studies [18–20] and review publications [21–24], we present the most useful instrumental methods used in the analysis of drug impurities.

8.4.1 Separative Methods

The first stage of impurity analysis is to separate the impurities from the active substance and excipients. For this, the following methods are used:

- Chromatography: TLC, HPLC, GC, supercritical fluid chromatography (SFC)
- Electrokinetic methods: capillary electrophoresis (CE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary electrochromatography (CEC)

The mentioned methods also allow identification and quantification of the impurities [19].

Currently, the most common method in pharmaceutical analysis is HPLC, which is also the most commonly used in impurity analysis, according to Niessen [25] and Olsen et al. [26]. Uses of HPLC are wide, and include examples such as:

- Identification and quantification of seven impurities in paracetamol [27]
- Identification of several impurities and degradation products of loteprednol etabonate [28]
- Use of columns with monolithic filling to separate acyclovir from guanine [29] and rifampicin from four impurities [30]
- Use of HPLC with oil/water emulsion introduced to the mobile phase (micro-emulsion liquid chromatography, MELC) for quantification of simvastatin [31] and five impurities of paracetamol [32]

In the mentioned examples, detection was performed with the use of a UV lamp. For drugs that do not absorb UV light, electroanalytical detectors can be used, such as an amperometric detector for analysis of neomycin sulfate [33] and amikacin [34] and their impurities.

Beside HPLC, TLC is also commonly used in pharmaceutical analysis. Although it is less selective and less precise than other chromatographic methods, its versatility, rapidity, and low cost make it a popular method. The traditional TLC method is gradually being replaced by HPTLC, which uses new stationary phases and automatic densitometric detection. HPTLC is a method competitive with HPLC in the analysis of drug impurities [19, 20].

Table 8.3 Electrokinetic methods for analysis of drug impurities

Active pharmaceutical ingredients	Analytical methods	References
Amoxicillin	Separation and determination using MEKC	[43]
Heroine	Determination of impurity profile using MEKC	[44]
Hydrochlorothiazide, chlorothiazide	Separation and determination using MEKC	[45]
Bromazepam	Determination of impurity profile using CE	[46]
Tetracycline hydrochloride	Determination of impurity profile using CE	[47]
Ibuprofen	Determination of impurity profile using CE	[48]

MEKC micellar electrokinetic chromatography, *CE* capillary electrophoresis

TLC has been used for the impurity analysis of ciprofloxacin, diclofenac, chlorpromazine, trifluoropromazine, doxepine, alprazolam, omeprazole, pantoprazole, tetracycline, and chlortetracycline [35–41]; other examples can be found in the work of Ferenczi-Fodor et al. [42].

As an alternative to chromatographic methods, electrophoretic methods can be used in establishing the impurity profiles of drugs. The methods include CE, MEKC, and MEEKC. One of most useful methods is CEC. This technique combines the high efficiency of CE with abilities of LC. Examples of the use of electrophoretic methods in drug impurity analysis are shown in Table 8.3 [43–48].

8.4.2 Spectroscopic Methods

The use of UV–vis and FT-IR spectroscopy in determining drug impurities without previous chromatographic separation is limited because of the low selectivity of both methods. In some cases, when the impurity has spectral properties that are very different from those of the active substance, the direct measurement of absorbance can give some useful data about the structure of the impurity. But, in many cases the impurity has a similar structure to the active substance and, therefore, their UV and IR absorption spectra are similar, and overlap with each other additively. This means that direct use of spectroscopic methods in drug purity analysis is limited [49].

Selectivity of the UV method can be increased by the use of spectra derivatives [50]. Derivative spectrophotometry is a chemometric method in which classic UV spectra (zero-order spectra) are differentiated with respect to wavelength before being analyzed. It is much more selective and precise than classic UV spectroscopy [50]. Examples of the use of the spectra derivatives method in drug purity analysis are shown in Table 8.4.

The most-used spectroscopic method in drug analysis is mass spectrometry (MS). It allows (depending on the ionization technique) measurement of impurities at the nanogram or picogram level; if an ion bombarding technique is used, even

Table 8.4 Examples of derivative UV spectrophotometry in analysis of drug impurities [50, 53–61]

Active pharmaceutical ingredients	Analytical methods	References
Aciclovir, diloxanide furoate	Determination of acyclovir and guanine (impurity) using 2nd derivative spectrophotometry Determination of diloxanide furoate and its degradation product with 3rd derivative	[53]
Aceclofenac	Determination of aceclofenac and diclofenac (main degradation product) using 3rd derivative	[54]
Amlodipine	Determination of amlodipine and photodegradation products using 3rd derivative	[55]
Ceftazidime (I), cefuroxime (II), cefotaxime (III)	Determination of I, II, and III and their degradation products using 1st derivative	[56]
Hydrochlorothiazide	Determination of hydrochlorothiazide and its degradation products (methoxyhydrothiazine, hydroxyhydrothiazine and 5-chloro-2,4disulfonamidoaniline) using 1st and 2nd derivatives	[57]
Linezolid	Determination of linezolid and its degradation products using 1st derivative	[58]
Meloxicam	Determination of meloxicam and its degradation products using 1st derivative	[59]
Omeprazole	Determination of omeprazole and its degradation products (sulfenamide and benzimidazole sulfide) using 1st, 2nd, and 3rd derivatives	[60]
Secnidazol	Determination of secnidazole and its degradation products using 1st derivative	[61]

sub-picogram levels can be determined [51]. If the analyte was not previously chromatographically separated, it is useful to use tandem mass spectrometry (MS/MS), which uses different ionization techniques. Use of MS in the analysis of spironolactone purity is shown in Fig. 8.3 [52].

8.4.3 Tandem Methods [62–80]

The best results in the analysis of organic drug impurities are obtained by the use of tandem (hyphenated) methods, which combine the separative properties of chromatographic/electrophoretic methods with the qualitative and quantitative abilities of spectroscopic methods. In practice, the most common combinations include the use of LC or GC with UV, NMR, or MS detection, or combinations of electrophoretic methods with MS detection (CE-MS, MEKC-MS, and CEC-MS).

The use of UV spectroscopy, mostly using a photodiode array, allows quantification of drug impurities that have a different retention time from that of the active substance. An example is use of HPLC-UV for determination of the impurity profile

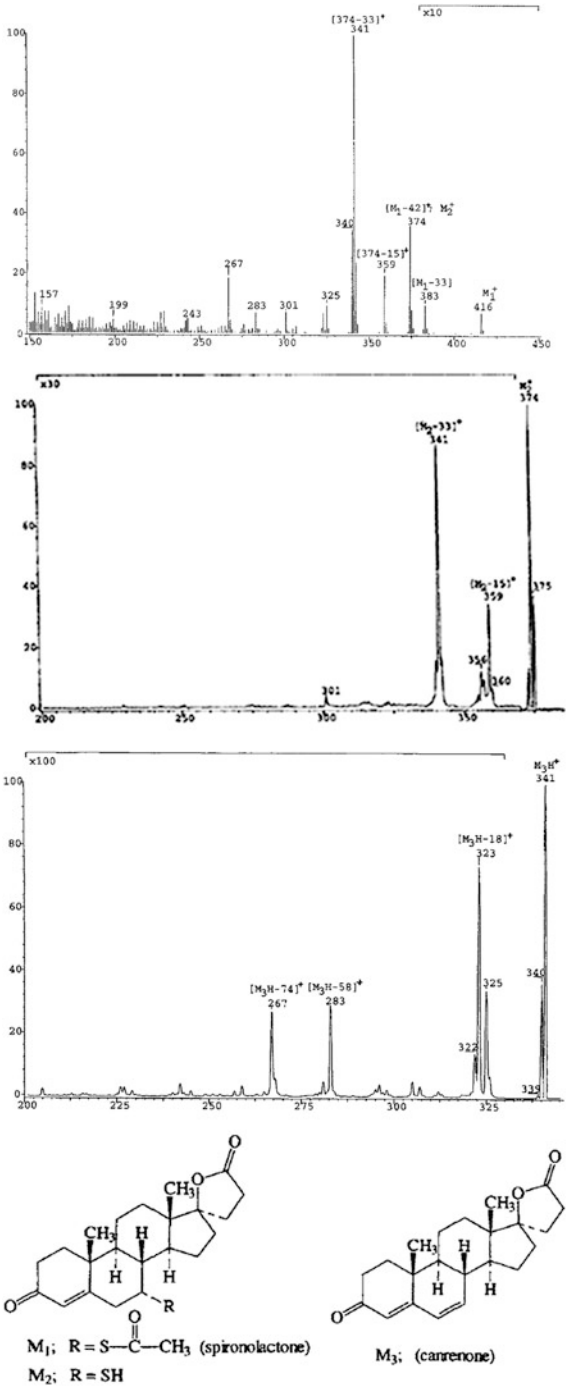

Fig. 8.3 MS spectra of spironolactone and its impurities [52]

of pipecuronium bromide, as presented by Görög [22]. This drug is not capable of absorbing electromagnetic radiation in the UV range, due to lack of chromophore, but its impurity (an enol derivative) absorbs UV radiation (with a maximum at 236 nm).

The use of UV detection in drug purity analysis was widely described by Zhou et al. [62], and also in the work of Görög [19].

Currently, the most commonly used method for determining drug impurities is HPLC-MS. Such analysis requires proper preparation of the sample, adequate adjustment of separation parameters, and use of an ionization method. The use of soft ionization produces the molecular ion of the impurity and enables its molecular weight to be established. Use of tandem MS/MS spectrometry allows the chemical structure of the impurity to be established by marking its fragmentation schemes. Liquid chromatography coupled with MS detection was used in purity studies of zaleplon [63], etoricoxib [64], ethanediol, diacetate [65], dicloxacillin [66], quinapril [67], and others described in several review articles [68–70].

The use of GC-MS in drug analysis is limited to drugs of appropriate thermal stability and volatility. Although use of HPLC-MS has decreased the importance of GC-MS in pharmaceutical analysis, improvements in the analytical process (e.g., introduction of high selectivity MS detectors) has meant that GC-MS remains an important method for analysis of drug impurities [71–74].

Tandem electrophoretic methods, such as CE-MS, MEKC-MS, and CEC-MS, can be used as alternatives to LC methods for analysis of drug impurities:

- CE-ESI-MS/MS method in the analysis of galantamine hydrochloride impurities [75]
- CE-ESI-MS method in the analysis of 16 impurities of potential medications [76]
- CE-MS method in the identification and quantification of *cis*-ketoconazole as a impurity of ketoconazole [77]
- MEKC-MS method in the analysis of ibuprofen and codeine phosphate [78]
- MEKC-APPI-MS method in the analysis of impurities of mebeverine [79]
- MEKC-ESI-MS method in the analysis of purity of galantamine hydrobromide and degradation products of ipratropium hydrobromide [80]

In conclusion, tandem methods with the use of different detectors of high efficacy are currently the best choice for analysis of drug impurities. They allow both identification and quantification of the impurities present in medications, even at such low levels as parts per million (ppm) or parts per billion ($1/10^9$, ppb).

8.4.4 Methods for the Analysis of Inorganic Impurities

The biggest group of inorganic impurities are metals, which are usually analyzed using atomic spectrometry techniques (e.g., atomic absorption spectroscopy, AAS;

Table 8.5 Limits for metals in pharmaceuticals [90]

Metal	Oral daily dose (PDE) (μg/day)	Component limit (μg/g)		Component limit (μg/g)		
		Oral	Parenteral	ICP-OES	GFAAS	ICP-MS
High toxicity						
Arsenic (inorganic)	15	1.5	0.15	3.5	0.1	0.01
Cadmium	25	2.5	0.25	0.06	0.0008	0.002
Lead	10	1.0	0.10	2.0	0.04	0.003
Mercury (Hg^{+2})	15	1.5	0.15	3.0	0.6	0.001
Intermediate toxicity						
Chromium III	250	25	2.5	0.3	0.05	0.02
Molybdenum	250	25	2.5	0.12	0.006	0.002
Nickel	250	25	2.5	0.6	0.05	0.02
Palladium	100	10	1.0	4.0	0.05	
Platinum	100	10	1.0	2.0	0.02	0.003
Osmium	100	10	1.0	2.0		0.001
Rhodium				2.0	0.01	
Ruthenium				5.0	1.0	
Iridium				2.0	0.05	
Vanadium	250	25	2.5	0.78	0.1	0.004
Low toxicity						
Copper	2500	250	25	0.2	0.001	0.01
Manganese	2500	250	25	0.05	0.005	0.02

PDE permitted daily exposure

inductively coupled plasma–atomic emission spectroscopy, ICP-AES) or mass spectrometry (e.g., ICP-MS) [19].

The most commonly used is AAS (flame and non-flame), which allows both the identification and quantification of metals in solid, liquid, and gas samples [81]. ICP-AES allows analysis of impurities at trace levels (nanogram and picogram traces) [81]. ICP-MS allows both detection and analysis of most of the elements of the periodic table, as well as quantification of the concentrations of different isotopes of a given element in different matrices (body fluids, water, sewage, etc.). The limits of detection for many elements are at the 10^{-10} to 10^{-13} ppm [81].

ICP-MS is one of the newest instrumental methods used in drug analysis, and has been used in the following:

- Investigation of drug metabolism: quantification of 4-bromoaniline metabolites in rat urine [82]
- Quantification of the active substance: vitamin B_{12} [83], cimetidine [84], drugs with heteroatoms (S, P, Cl, Br, I) or metals [85], such as cisplatin [86]
- Analysis of radiopharmaceuticals: quantification of ^{99}Tc to ^{99m}Tc ratio with quantification limit of 50 pg/mL [87]

Table 8.6 Examples of limit of quantification and detectors in analysis of inorganic impurities [91–95]

Drugs	Elements	Limit of quantification	Detector	References
Dicyclomine, ethambutol, pyrzinamide, furazolidone	Ti, Cr, Mn, Co, Ni, Cu, Zn, Cd, Hg, Pb	-	Varian Ultra Mass 700	[91]
Neusilin	Al, Mg	40 and 6 μg/g	CETAC LSX-100, Perkin-Elmer SCIEX, ELAN 6000 ICP-MS	[92]
Vitamin E	Ti, V, Cr, Mn, Fe, Co, Cu, Mo, Ag, Cd, Ni, Sn, Pb	0.01–3.02 ppb (emulsion), 0.01–1.26 ppb (15 % HNO_3)	Agilent7500 ICP-MS, 1100 W, Meinhard Nebulizer	[93]
Methamphetamine hydrochloride	Na, Br, Pd, Ba, I	-	Seiko ICP-Ms SPQ-6100, 1.35 kW quadrupole type	[94]
Fosinopril sodium	Pd	0.1 μg/g	Plasma Quad PQ 11 Turbo plus ICP-MS Jacketed Scott Type Spray Chamber	[95]

– Biomedical analysis: analysis of DNA, proteins, quantification of P, Zn, Cu, and Fe levels in Alzheimer's and Parkinson's diseases, toxicity of elements [88, 89]

Table 8.5 presents the results of the identification of 16 elements present in drugs using GF-AAS, ICP-OES, and ICP-MS methods. Table 8.6 presents examples of uses of ICP-MS for analysis of elements present in drugs.

8.4.5 Methods for Analysis of Volatile Solvent Residues

Volatile solvent residues in drugs are leftovers of organic compounds used in the synthesis of either the active substance or the final formulation. Organic solvents play an important role in the pharmaceutical industry, but unfortunately many of them are toxic for humans and for the environment. Total removal of these solvents from final products is not always possible, which is why there is a need to establish their profiles [90].

Thermogravimetric analysis (TGA) can be used in the analysis of solvent residues in rugs. TGA measures the change in sample weight with time and change in temperature. It allows both identification and quantification of solvents, with a quantification limit of 100 ppm [96].

Table 8.7 Summary of common gas chromatographic detectors used in residual solvent analysis [97]

Detector	Type	Detection limit (g)
Thermal conductivity detector (TCD)	Universal	4×10^{-10}
Flame ionization detector (FID)	Universal (organic/carbon compound)	2×10^{-12}
Photoionization detector (PID)	Universal	2×10^{-13}
^{63}Ni electron capture detector (ECD)	Selective (halogens and other electron-withdrawing groups)	5×10^{-15}
Mass spectrometer (MS)	Universal or selective	To 25×10^{-15}

IR methods can be used for quantification of tetrahydrofuran, dichlorobenzene, and methylene chloride [96].

The most useful method for solvent residue analysis is GC. It can be performed by direct injection technique, or by headspace, solid phase microextraction (SPME), or single-drop microextraction (SDME) techniques [96]. GC has high selectivity, good specificity, is easy to perform, and involves simple sample preparation. Modern capillary GC allows separation of many compounds, together with their identification and quantification [96]. GC uses different detector systems, which are presented in Table 8.7.

8.5 Summary

In the modern world, trace analysis plays an important role in such areas as ecology, chemical engineering, food processing, biomedical analysis, and drug chemistry. Advanced, sensitive, and precise analytical methods allow identification and quantification of different compounds, both organic and inorganic, including macromolecular complexes. All these methods present very good detection and quantification levels, up to parts per trillion (ppt) or parts per quadrillion (ppq).

Trace analysis is very important in the analysis of impurities present in the active substances of drugs and in final pharmaceutical preparations. Identification and characterization of the impurity profile of drugs is regulated with international standards, and is used to ensure the quality of pharmaceutical products.

This publication presents the categories of impurities according to ICH standards [7–9], requirements for different impurities, and analytical methods used in the characterization of the impurity profile of drugs. The review clearly shows the wide range of methods used in quality assurance for drugs. The continual development of these methods assures that the quality of drugs is constantly improving, which increases the safety of pharmacotherapy.

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Chapter 9
Elemental Trace Analysis in Studies of Food Products

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9.1 Introduction

Mineral elements play a key role in the human body and especially in the regulation of cell metabolism. They are either incorporated into the tissues or else are present in body liquids in ionic form. They also participate in metabolic processes such as electrolyte and hormone economy, haematopoiesis, and development of the nervous and skeletal systems [1, 2].

An imbalance in mineral component economy leads to disturbances in cell functions and the appearance of disease symptoms. The human-transformed natural environment usually deteriorates and exerts a negative influence on our health and food quality. The presence and availability of some mineral elements vital for the proper functioning of the human body are particularly vulnerable to such adverse changes. Diet and the chemical elements in food can affect the risk of contracting diseases to various extents. An inappropriate diet is a factor favouring the development of circulatory diseases and carcinomas [3] (the main causes of premature death) and has become a serious social and economic problem.

Trace amounts of toxic metals are detected in most foods as a result of environmental pollution caused by human agencies [4]. Nowadays, people have a much greater awareness of the effects that the food they consume have on their health and well-being. Food quality monitoring has thus become a basic task for both governmental organisations and food producers. Analytical procedures have to be updated continuously in response to technological advances so that the quality of food products can be precisely determined. Food products can be analysed for their elemental content with respect to daily amounts consumed; this enables the quantities of metals ingested with the diet to be measured and, consequently, the danger

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of being poisoned by toxic elements to be identified. Analysis of the elemental content of food also indicates whether the product in question has been contaminated by environmental or technological pollutants. The following elements are usually analysed in food: Ca, Mg, Na, K, P, Zn, Cu, Mn, Fe, Cr, Co, Ni, Se, Sn, Mo, As, Cd, Pb and Hg.

Maintaining the quality of food is a far more complex problem than the quality assurance of non-food products. Analytical methods are an indispensable monitoring tool for controlling levels of substances essential for health and also of toxic substances, including heavy metals. The usual techniques for detecting elements in food are flame atomic absorption spectroscopy (FAAS), graphite furnace atomic absorption spectrometry (GF AAS), hydride generation atomic absorption spectrometry (HG AAS), cold vapour atomic absorption spectrometry (CV AAS), inductively coupled plasma atomic emission spectrometry (ICP AES), inductively coupled plasma mass spectrometry (ICP MS) and neutron activation analysis (NAA).

9.2 Preparation of Samples for Analysis

A key aspect of every analytical procedure is the proper preparation of samples [5]. The determination of a target analyte in a sample begins with taking a suitable weighed amount, known as an analytical sample. The magnitude of this weighed amount depends on the quantity of food product available and also on the estimated content of the target analyte [6]. In addition, the sample must be representative; that is to say, the sample should be taken, prepared and stored in such a way that its chemical composition resembles as closely as possible that of the food product being analysed [6].

The material to be analysed should always be homogenised before samples are weighed; if samples contain water or are heterogeneous, they do not adequately reflect the structure of the material under scrutiny [7]. Homogenisation procedures depend on the physico-chemical form of the food product. It may have to be cut, mixed, chopped or crushed to obtain a homogeneous mixture from which an analytical sample can be taken [8]. The procedure for preparing samples for trace analysis has been described in detail by Hoenig [9]. Food products to be homogenised can become contaminated by the equipment used for this purpose [8]. Examples of such contamination have been described, inter alia, by Gouveia et al. [10] and Cubadda et al. [11]. The latter authors found a number of elements (Al, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb) that were responsible for contamination of samples.

Once weighed, the analytical sample must be completely solubilised by means of mineralisation. This process involves the decomposition and oxidation of any organic compounds contained in the sample and their conversion into inorganic derivatives. There are two main types of mineralisation: dry and wet.

Dry Mineralisation Dry mineralisation techniques include incineration (ashing), low-temperature mineralisation in oxygen plasma, mineralisation in oxygen, and melting.

Incineration is the usual method in food analysis. Organic matter is decomposed in an electric furnace within a temperature range of 400–600 °C. It is rather a long procedure and the ash it yields, consisting mostly of carbonates and oxides, is dissolved in a suitable acid or mixture of acids [12–16].

Mader et al. [17] reviewed the procedures used for dry mineralisation. They showed that it is usually carried out at temperatures of 450–500 °C with the addition of magnesium nitrate. Vassileva et al. [18] found that adding $MgNO_3$ to the mineralisation mixture reduced losses of As and Se. Fecher and Ruhnke [19] demonstrated that cross-contamination with Cd and Pb was possible during dry mineralisation without any additives at 450 °C.

Food products that are solubilised using this technique include coffee [13], confectionery [20, 21], condiments [22], meat products, fruit and vegetables, and cereal products [12, 20].

Wet Mineralisation Wet mineralisation techniques can be classified according to the source of heat energy:

- Conventional heat sources
- Ultrasound
- UV radiation
- Microwaves

These techniques are also classifiable according to the type of system in which they are performed:

- Open systems
- Closed (high-pressure) systems
- Stationary systems
- Flow-through systems
- Gaseous phase systems

The matrix is decomposed with the aid of one or more strong mineral acids, with or without the addition of other oxidising compounds. These oxidising agents are usually concentrated acids (HNO_3, H_2SO_4, $HClO_4$) and H_2O_2 [6, 16, 22]. This technique has been used to dissolve food products such as fish and seafood [23–25], mushrooms [26], cereal products, dairy products, meat products, fruit and vegetables [27, 28], and condiments [22].

Table 9.1 lists examples of mineralising mixtures and heating systems used in wet mineralisation, depending on the target element and the material analysed.

Heating in a Convectional System This procedure is based on decomposition of the sample using convectional heating and oxidising acids. A drawback of this system is that samples cannot be prepared for the detection of volatile substances; advantages are that it is inexpensive and mineralisation takes place quite quickly [5]. Tinggi et al. [37] compared three mineralisation procedures using mixtures of

Table 9.1 Wet mineralisation techniques

Mineralisation technique	Type of material	Reagents required	References
Open systems			
Convectional heating	Plant material and mushrooms	HNO_3, H_2SO_4, $HClO_4$, H_2O_2	[29]
Microwave heating	Krill	HNO_3, H_2O_2	[30]
UV radiation mineralisation	Sea water	H_2O_2	[31]
Closed systems			
Convectional heating	Fish	HNO_3	[32]
Microwave heating	Seafood	HNO_3	[25]
Flow-through systems			
Convectional heating	Blood serum	HNO_3, H_2SO_4, $HClO_4$	[33]
UV radiation mineralisation	Water, oysters	HNO_3, H_2O_2	[34]
Microwave heating	Human blood	HNO_3	[35]
Gas-acid phase mineralisation	Biological material	HNO_3	[36]

acids (HNO_3–$HClO_4$–H_2SO_4, HNO_3–H_2SO_4 and HNO_3–H_2SO_4 with added HF). They showed that adding HF was not necessary to obtain satisfactory Mn and Cr determinations using GF AAS on a wide assortment of food products. Muñoz et al. [38] used this technique to determine As in the daily food rations of the inhabitants of Santiago (Chile).

Microwave Heating The standard technique for solubilising samples is high-pressure mineralisation using microwave energy. The idea is to react the sample with oxidising acids at an elevated temperature in a Teflon bomb. The pressure resulting from the evolution of gases enables higher temperatures to be achieved than the boiling points of the acids in open systems, and this shortens the reaction time [5].

The advantages of microwave mineralisation are [8, 39]:

- Good repeatability
- Simplicity of operation
- Short reaction time
- Minimal consumption of reagents
- Determination of a large number of elements over a wide range of concentrations
- Limited analyte losses
- Minimal sample contamination
- Effective decomposition of organic and inorganic substances

The drawbacks of this technique are [8, 39]:

- Expensive apparatus
- Only small weighed amounts of sample can be used

At present this is one of the most common techniques for mineralising food samples, more than adequately replacing traditional techniques. Lamble and Hill [40] reviewed the procedures used with this technique. Sun et al. [41] applied three wet mineralisation procedures using mixtures of HNO_3, HNO_3–H_2O_2 and HNO_3–H_2O_2–HF to prepare samples of reference materials based on food products to determine the content of 13 elements (Al, B, Ca, Cu, Fe, K, Mg, Mn, Na, P, S, Sr, Zn). Mineralisation with HNO_3–H_2O_2–HF yielded satisfactory results for all except three materials, for which only the results for Al were acceptable. Gawalko et al. [42] applied microwave heating in open and closed systems to solubilise samples of cereal products, achieving satisfactory determinations of Cd, Cu, Pb and Se. Dolan and Capar [43] developed a wet mineralisation procedure using microwave heating, based on selecting the size of the food sample to be analysed (from 0.41 to 9.5 g) in accordance with the energy value of the target food product. Krzysik et al. [44] used microwave mineralisation to determine the Cr content in fast-food products and convenience foods, whereas Kwoczek et al. [25] used the same technique to determine both essential elements and toxic metals in samples of seafood.

9.3 Determining the Content of Selected Elements in Food Products

Flame Atomic Absorption Spectroscopy FAAS is one of the oldest analytical techniques and continues to be used in the analysis of food products. The analysis is usually performed in an air–acetylene or a nitrous oxide–acetylene flame. The technique measures the absorbance of electromagnetic radiation by the free atoms produced at high temperature (1000–4000 K) [6].

FAAS is used to determine many essential elements and toxic metals in food products. It is quick and offers a satisfactory level of selectivity, accuracy and sensitivity. According to AOAC, the standard methods based on this technique include the determination of Cd [45] and Pb [46] in food, Cu, Fe and Zn in food [47, 48], Sn in canned products [49], Zn in food [50], Zn [51], Cu [52] and Fe [53] in fruit and vegetables, Zn in milk [54], Ca and Mg in cheese [55], and Ca, Cu, Fe, Mg, Mn, K, Na and Zn in specialist food products [56]. Table 9.2 lists some applications of FAAS in the analysis of various food products. Grembecka et al. [13] used FAAS to determine the amounts of certain essential elements and toxic metals in coffee and confectionery products. Milacic and Kralj [68] also used it to determine levels of Zn, Cu, Cd, Pb, Ni and Cr in food products available in Slovenia. Another example of the application of FAAS is the analysis by Khajeh [69] of Zn and Cd after prior extraction. Santelli et al. [64] assessed the degree to which the mineralisation of food samples was effective. Miller-Ihli [70] employed FAAS to assess adherence to legal regulations governing the labelling of food products (Ca, Cu, Cr, Fe, Mg and Zn). With the aid of FAAS and statistical methods for

Table 9.2 FAAS application for the quantitative analysis of elemental composition of food products

Food	Elements	References
Diet supplements	Cd, Fe, Pb, Zn	[57]
Total diet	Ca, Cu, Mg, Na, P, Zn	[58]
Coffee	Cd, Pb, Co, Cr, Cu, Mn, Fe, Ni, Zn, Ca, Mg, Na, K	[13]
Seafood, meat, cheese, cereal products, legumes, dry fruits	Ni	[59]
Vegetables	K, Na, Ca, Mg, Fe, Mn, Cu, Zn	[60]
Vegetables	Cd, Cu, Ni, Pb, Zn	[61]
Canned food	Cu, Zn, Mn, Fe	[62]
Baby food	Cu, Zn, Mn, Fe	[63]
Cereal products	Fe, Zn, Mn	[64]
Fruits, vegetables, legumes	Ca, Cu, K, Mg, Mn, Zn	[65]
Black tea	Cu, Ni, Cr	[15]
Beer	Fe, Mn, Zn, Cu, Mg, Ca, Al	[66]
Raw and processed food	Zn, Fe, Ca	[67]
Seafood	Co, Cr, Cu, Mn, Fe, Ni, Zn, Ca, Mg, Na, K	[25]

interpreting measurement data, it was possible to distinguished coffees grown organically and in the traditional way [71].

Graphite Furnace Atomic Absorption Spectrometry GF AAS is based on absorption by free atoms, of the resonance lines characteristic of a given element, emitted by the radiation source [6, 72].

With this technique both essential and toxic elements in food can be detected at lower levels than is the case with FAAS. GF AAS is highly sensitive and selective, and samples can be analysed without prior preparation. Microsamples can be analysed, and the limit of detection is of the order of micrograms per litre. The drawbacks of GF AAS include poor repeatability, the determination of only single elements, and matrix effects, which can significantly lower the quality of the analytical results [72].

Standard methods using this approach include the determination of Cd and Pb in food [47, 48]; Cd [73] and Pb [74] in fruit and vegetables; Sn in canned condensed milk [75]; Cd in oils and fats [76]; Pb [77], P [78], Cu, Fe and Ni [79] in plant and animal fats; Cd [80] and Pb [81] in starchy products; and Pb in sugar and syrups [82].

Table 9.3 lists some applications of this technique in food analysis. Julshamn et al. [95] developed a method of microwave mineralisation in a closed system for the GF AAS determination of As in seafood. They found the technique to be sufficiently sensitive to determine As at the level of 2.5 mg/kg dry mass. In contrast, Fedorov et al. [96] compared GF AAS with HG AAS in determinations of As and

Table 9.3 Application of GF AAS in food analysis

Food	Elements	References
Total diet	Cd, Pb, Ni	[83]
Duplicate diet, fruit juices	Al	[84]
Food, convenience products	Cd, Cr, Ni, Pb	[85]
Meat, milk, vegetables, cereals	As, Cd, Pb	[86]
Cereals products, vegetables, fruits, meat, fish, eggs, beverages	Cd, Pb, Ni, Se	[87]
Meat, seafood, dairy and cereal products, vegetables, fruits, oils and fats, nuts	Cr	[88]
Seafood, vegetables, olive oil, dairy products, energy drinks, fruit juices, soft drinks	Al	[89, 90]
Legumes, nuts	Cu, Cr, Fe, Zn, Al, Ni, Pb, Cd	[91]
Oils	Al, Cu, Co, Cr, K, Ni, Mn, Pb	[92]
Canned food	Se, Al, Cr, Ni, Co	[62]
Seafood, cereal and dairy products, vegetables, olive oil	Cr	[93]
Condiments, nuts, cereal products, coffee, baby foods, vegetables and dried fruits	Pb, Cd	[94]

found that better accuracy and greater precision were obtainable with the latter technique. Veillon and Patterson [97] drew attention to a number of analytical difficulties that the analyst has to face when determining Cr using GF AAS.

Hydride Generation Atomic Absorption Spectrometry HG AAS is a method that makes use of the ability of such elements as As, Bi, Ge, Pb, Sb, Se, Sn and Te to form volatile compounds with hydrogen.

HG AAS is normally used to determine As and Se in food products [98–101]. The preparation of samples for analysis usually involves their wet mineralisation in a mixture of HNO_3–$HClO_4$–H_2SO_4 or their incineration with magnesium nitrate and magnesium oxide. Kabengera et al. [102] developed a fairly simple HG AAS technique useful for determining As in a mineralising mixture of HNO_3–H_2O_2. To determine Se in meat products using HG AAS, Tinggi [103] suggested mineralisation in a mixture of concentrated HNO_3–$HClO_4$–H_2SO_4.

HG AAS has been used to determine As and Se levels in whole food rations [83, 104–196], meat [107], selected food portions [108], seafood [109], soya milk and baby food [110], as well as in cereal grains [111], coffee, fish and oysters [112].

Cold Vapour Atomic Absorption Spectrometry CV AAS is the best and most sensitive method for determining very low levels of mercury; it makes use of the fact that atoms of this element can exist in gaseous form at room temperature.

Standard methods based on CV AAS are used to determine Hg and other elements in fruit and vegetables [113] and in fish [114]. CV AAS has also been used to detect Hg in daily food rations [15, 83, 106], food products [86, 87], mushrooms [116] and seafood [115, 117–121].

Dabeka et al. [122] developed a method for determining low levels of Hg in food products using low-temperature mineralisation. Larsen et al. [87], on the other hand, applied high-pressure incineration and CV AAS to determine Hg in a broad assortment of food products. Zenebon et al. [123] mineralised food samples for Hg determination using a mixture of H_2O_2 and H_2SO_4 in open vessels heated in a water bath to 80 °C. These authors describe their method as simple, quick (mineralisation is completed overnight), sensitive and inexpensive [8].

Inductively Coupled Plasma Mass Spectrometry ICP MS yields analytical measurements of great accuracy and low limits of detection [124]. The advantages of ICP MS are as follows [16, 124, 125]:

- Analysis of one or many elements at the same time is feasible, as is the determination of elements with high excitation potentials (e.g. W, Cl, Br, I, S, U)
- Wide range of measurement linearity, corresponding to a concentration differentiation of four to five orders of magnitude
- Determination of both principal and trace components in the same sample is possible
- No contamination, because electrodes are not used for excitation
- Low limit of detection: 0.01–10 μg/L
- Excellent sensitivity and selectivity
- Analysis is quick and straightforward
- Little interference from matrix effects

The drawbacks of ICP MS [16, 124] are the following:

- Substantial dilution limits the use of the method because suitably low levels of detection and quantification are not achievable
- Interferents have to be removed during the measuring cycle
- Reduction or elimination of matrix effects is necessary
- Acids of very high purity have to be used
- Analysis is very costly
- Argon and other reagents have to be of very high purity
- Samples with a complex matrix have to be solubilised
- Samples have to be diluted

Table 9.4 lists the possible uses of ICP MS in food studies. Zbinden and Andrey [126] developed a routine method for determining Al, As, Cd, Hg, Pb and Se in food using ICP MS following prior high-pressure incineration. They dealt with interferents by adding isopropanol to the analyte solution. Bhandari and Amarasiriwardena [127] described a method for determining Pb and seven other elements in maple syrup using ICP MS following wet mineralisation in a microwave system. ICP MS was also used to detect I in baby food products [128] and in dietetic products [129]. Choi et al. [130] attempted to define Se levels in samples of food from South Korea following their prior mineralisation with HNO_3 in a convection system. Nardi et al. [28] implemented ICP MS to determine Se, Cu, Zn, Co and Mn in food products, including meat, cereal products, dairy products and vegetables.

Table 9.4 Application of ICP MS in food analysis

Food	Elements	References
Seafood	CH_3Hg, Zn, As, Se, Cd, Hg, Pb	[136]
Total diet	As, Cd, Pb	[137]
Total diet	Br, I	[138]
Duplicate diet	Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sn, Zn	[139]
Onion, peas	Ag, Al, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Ho, In, Ir, K, La, Li, Lu, Mn, Mo, Na, Nb, Nd, P, Pb, Pr, Pt, Rb, Re, Ru, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Tb, Te, Th, Ti, Tl, Tm, U, V, W, Y, Yb, Zn, Zr	[140]
Orange juice	Ba, Co, Cu, Li, Lu, Mn, Mo, Ni, Rb, Sn, V, Zn	[141]
Edible wild mushrooms	Ag, Rb, Cd, Hg, Pb, Cs, Sr, Tl, In, Bi, Th, U, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La, Lu, Zn, Fe, Cu, Mn, Ba	[142]
Rice	Al, Ca, Mg, P, Na, K, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, Cd, Pb, B, Se, Rb, Gd, Ho, W	[143]
Black and green olives	Mg, Cr, Co, Ni, Fe, Cu, Zn, Sn, Cd, Pb	[144]
Canned vegetables	Mg, Al, Cr, Mn, Fe, Ni, Zn, Cu, Mo, Cd, Sn, Sb, Ba, Pb, Bi	[145]
Wine	Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Ag, Cd, In, Cs, Ba, Hg, Tl, Pb, Bi, U	[146]

Whyte et al. [131] availed themselves of ICP MS to assess the nutritional quality of mussels and the exposure of New Zealanders to the toxic effects of elements such as Hg, Sn, As, Cd and Pb. In view of the constant danger of Americans being exposed to lead, Bagga and Jarret [132] attempted to determine the levels of this element in vegetables cultivated in greenhouses in the USA. They found considerable amounts of this metal to have accumulated in cabbage, lettuce and turnips harvested from land exposed to a medium degree of pollution. Lead, antimony and mercury were the subject of a study by Chung et al. [133], who assessed exposure levels to these elements in Hong Kong school pupils. Jorhem et al. [134] tested a series of food products from Sweden for their levels of As (ICP MS) and Pb and Cd (GF AAS). Leblond et al. [135], on the other hand, tested dietary supplements of marine origin for their levels of Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn. Apart from Hg (mercury analyser) and Cr (ICP AES), all the other elements were determined using ICP MS.

Other Analytical Techniques Inductively coupled plasma optical emission spectrometry (ICP OES) or ICP AES can be used to simultaneously determine an average of 70 elements in a wide diversity of matrices. The benefits of this technique are as follows [16]:

- Analysis of one or many elements is possible, as is the determination of elements with high excitation potentials (e.g. W, Cl, Br, I, S, U)
- Wide range of measurement linearity, covering four to five orders of magnitude of concentrations
- Principal as well as trace components can be determined in the same sample
- No contamination (electrodes are not used for excitation)
- Low limit of detection: 0.01–10 μg/L
- Use of a polychromator enables determination of about 60 elements within a few minutes
- No self-absorption
- No interference as a result of the presence of oxygen

The disadvantages of ICP OES are:

- High cost of analysis
- Need to apply argon and other reagents of a very high grade of purity
- Differences in limits of detection for particular elements are several orders of magnitude (which hampers multi-elemental analysis)
- Spectral interference for elements rich in emission lines (e.g. U, W, Co, Fe)
- Routine operations and the handling of apparatus must be carried out by highly qualified persons
- Interference occurs between elements of similar wavelengths
- Ambient temperature and humidity need to be monitored
- Very good monochromatisation is required
- Determination of Group 1 metals is difficult

Since multi-elemental analysis is possible, ICP AES is widely used for food testing. It is the standard technique for determining Ca, Cu, Fe, Mg, Mn, P, K, Na and Zn in baby foods [147]; B, Ca, Cu, K, Mg, Mn, P and Zn in plant matter [148]; and P in vegetable and animal fats [149]. Miller-Ihli [150] demonstrated the applicability of ICP AES in the testing of food for the presence of Ca, Co, Cu, Cr, Fe, Mg, Mn, Ni, P, V and Zn following wet and dry mineralisation of samples. Dolan and Capar [43] used ICP AES to determine 20 elements in food products. Lomer et al. [151] developed a method using ICP AES for detecting Ti in food, with the aim of determining levels of the additive titanium dioxide. This method involved the mineralisation of samples with H_2SO_4 heated to 250 °C. Rao et al. [152] used ICP AES to determine levels of Na, K, Ca, Mg, B, Pb, Cr, Co, Fe, Zn, Mn, Hg, Cu, As, Ni, Cd and Mo in the edible seaweed *Porphyra vietnamensis*, following wet mineralisation. The results of these analyses confirmed the utility of this product as a dietary supplement because of its beneficial mineral profile. The application of ICP AES, quadripole (Q) ICP MS and high-resolution (HR) ICP MS in combination with wet microwave mineralisation enabled levels of contamination by As, Cd, Co, Cr, Cu, Fe, Mn, Pb, V and Zn to be assessed in samples of rice [153]. Table 9.5 lists examples where ICP AES has been used to analyse food products.

Table 9.5 Application of ICP AES in food analysis

Food	Elements	References
Total diet	Ca, Cu, Fe, Mg, Mn, K, P, Na, Zn	[83]
Wild mushrooms	Al, Ca, K, Mg, Na, P, Si	[142]
Orange juice	Al, B, Ca, Fe, Mg, P, K, Si, Na, Sr, Ti	[141]
Tea	Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Sr, Zn	[155]
Soluble coffee	Na, K, Mg, Al, P, S, Ca, Mn, Fe, Ni, Cu, Zn, Cd, Sb, Pb, Cr, Sn	[156]
Oil	Ca, Fe, Mg, Na, Zn	[92]
Condiments	Al, B, Ba, Bi, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mn, N, Na, P, Pb, S, Se, Sr, V, Zn	[157]
Instant soups and seasoning mixtures	Na, K, Ca, Mg, P, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn	[158]
Aniseed drinks	Zn, B, Fe, Mg, Ca, Na, Si	[159]
Honey, confectionery products	Pb, Cd, Cu, Cr, Co, Ni, Mn, Zn	[160]

Neutron Activation Analysis This technique permits the quantitative and qualitative identification of elements. It is based on the conversion of a stable atomic nucleus into a radioactive nucleus by bombarding it with neutrons. The radiation emitted by the radioactive nuclei is then measured [154]. The advantages of NAA include [16, 154]:

- Indestructibility
- Excellent sensitivity
- Some 50–65 elements can be determined simultaneously
- Low limit of detection, of the order of micrograms per kilogram
- Preliminary sample preparation is not required

The drawbacks of this technique are as follows [16, 153]:

- Labour intensive
- Time consuming
- Overall level of an element can be determined without any differentiation of its chemical form and/or physical state
- Some elements (e.g. Pb) cannot be determined
- Access to a nuclear reactor is required
- High investment costs

Kucera et al. [161] performed a comparative analysis of three procedures for determining I in food. Their accuracy and precision were checked against reference materials based on food products. The limit of detection was 1 μg/kg. Instrumental NAA (INAA) and replicate sample INAA (RSINAA) were used in Portugal to test a wide assortment of food products for their total Se content [162]. INAA and radiochemical NAA (RNAA) were also used to assess the quality of bottled water sold in Greece [163]. Elements such as U, Ba, La, Sb, Ca, Cr, Zn, K, As, Br, Se and

Table 9.6 Application of NAA in elemental analysis of food products

Food	Elements	References
Total diet	Ca, Co, Cr, Cs, Fe, I, K, Se, Sr, Th, Zn	[165]
Total diet	Al, As, Br, Ca, Cl, Co, Cr, Cs, Fe, I, K, Mg, Mn, Na, Rb, Sc, Se, Sm, Sr, Th, U, Zn	[166]
Duplicate diet	Br, Ca, Cl, Co, Cr, Cs, K, Fe, Mn, Mg, Mo, Na, Rb, Se, Zn	[167]
Food	Ca, Cl, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Se, Sn, Zn	[168]
Traditional Mexican food	Fe, Al	[169]
Cereal products	Hg, Se	[170]
Cereal products, vegetables, condiments	Na, K, P, Cl, Br, Co, Cr, Cs, Cu, Fe, Hg, Mn, Mo, Rb, Sb, Sc, Se, Sr, Th, Zn	[171]

Co were determined in the dry residues left after the evaporation of samples on a water bath [163]. Islam et al. [164] used NAA to determine As levels in water, soil and plant matter. Table 9.6 lists the applications of NAA in the analysis of food.

9.4 Speciation Analysis of Elements

Speciation refers to the different physical and chemical forms of an element in a sample. Speciation analysis involves identifying the physical and chemical forms of an element and their determination in a sample.

Most legal regulations in force worldwide are based on overall levels of elements in food products; only a few concern particular forms of elements. Organisations such as the Environmental Protection Agency (EPA), World Health Organisation (WHO), European Environmental Commission (EEC) and US Food and Drug Administration (USFDA) have recognised the importance of speciation analysis and proposed limits to contamination by various forms of elements (e.g. As, Hg and Sn) [8, 172].

Determination of the speciation forms of elements occurring in different oxidation states(e.g. Cr, Fe, As and Se), as well as the various forms of organometal compounds (e.g. tributyltin, methylmercury, arsenobetaine), presents a serious challenge to analysts and is an increasingly common research topic worldwide. Because speciation forms are usually labile and occur in various matrices at very low concentrations (e.g. micrograms per kilogram), both sensitive and selective techniques are needed to determine them [173]. The standard analytical techniques used in speciation analysis are chromatographic methods (GC, HPLC) coupled with a wide range of detectors, including AAS, flame photometric detector (FPD), ICP AES, ICP MS and atom fluorescence spectroscopy (AFS). ICP MS in combination with different separation techniques, such as LC, GC, supercritical fluid chromatography (SFC) and capillary electrophoresis (CE), is also commonly used for this

purpose [136, 174–178]. Vieira et al. [179] reviewed all the techniques applied in speciation analysis except chromatographic methods. They explored a range of extraction procedures, including single drop microextraction (SDME), as well as derivatisation techniques. The analytical techniques used for analysis of the various elemental species in drinking water and food samples are suitably selective and sensitive [180–185].

Highly advanced analytical techniques are widely applied in the testing of environmental samples, including food, especially in the quantitative analysis of the speciation forms of both essential and toxic elements, including Fe, Zn, Cu, Co, V, Pt, Ag, Ca [186–192], As [185, 193–202], Sb [203–205], Mn [206], Cr [185, 187, 207–211], Hg [120, 185, 212], Se [185, 213, 214], Cd [187, 188, 215–217], Pb [187, 218–225], Ni [187, 188, 226], Tl [227], Sn [172, 228, 229], Te [230], S, N, P, Cl, I [231, 232] and Al [233].

The consecutive steps in speciation analysis are sample collection, sample conservation and storage, elimination of counterfeit samples, extraction/mineralisation, derivatisation, separation, detection, calibration, calculation of results and evaluation of results [212, 234, 235].

The application of speciated isotope dilution mass spectrometry (SIDMS) in speciation analysis was described and discussed by Huo et al. [236]. According to Encinar [237], isotopic analysis of a solution can be used to evaluate the application of various speciation forms of elements in food and in medications.

From the analytical point of view, certified reference materials play a vital role in food monitoring, as they permit quality and measurement accuracy to be assessed. The need to apply such materials in monitoring the quality of speciation analysis measurements was justified by Quevauviller [182] and Emons [238].

Arsenic Arsenic is a metalloid that can occur in both organic and inorganic forms: these differ markedly in their toxicity. Organic compounds of arsenic (e.g. arsenocholine and arsenobetaine), which occur mainly in seafood, are regarded as non-toxic [172, 189, 239], but the inorganic forms of this element are strong poisons and can be carcinogenic [240–246]. Because the metabolic changes As undergoes in marine organisms are still the subject of many studies, it is essential to determine not just the total content of As but also that of its various speciation forms. For example, fish contain high levels of As, but most of it is in organic form. Thus, consumers who eat large quantities of fish do not run the risk of As poisoning. However, no data are available on the toxicity of arsenosugars or on other organic compounds of this element, so the potential risks cannot be evaluated. Consequently, simple, validated analytical methods, as well as relevant regulations concerning As based on speciation analysis, are essential if the quality of fish and their products are to be properly assessed [247].

The literature reports many analytical techniques used for the speciation analysis of As in multifarious matrices, including food. The usual methods are GC or LC coupled with spectroscopic or electrochemical detection [185, 248]; the standard detectors are AAS, ICP AES and ICP MS [192, 246, 249]. One of the more favoured techniques for the speciation analysis of As is HG AAS coupled with

LC [172]. The speciation forms of this element have been determined, for example, in oysters and seaweeds [250–252], beer [253], water and other food products [185], rice [178, 254–256], fish oil [257, 258] and baby food [259].

Speciation forms of As such as As(III), As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (TMI) have been determined using cation- and anion-exchange chromatography coupled with ICP MS. For example, Wrobel et al. [248] used HPLC + ICP MS to determine the various forms of As in fish tissues. This same rapid and sensitive technique was used for As species in edible tissues of mussels and to monitor measurement quality [260]. A technique utilising anion-exchange gradient chromatography coupled with ICP MS was applied to detect As species in lobster tissues [261]. Ion-exchange chromatography combined with HG AAS was used to determine speciation forms of As in commercially available seafood [172]. Chatterjee [250] determined various forms of As in edible tissues of oysters using microwave wet extraction and HPLC + ICP MS. The proportions of various forms of As in seaweed and oysters were determined with multidimensional liquid chromatography with ICP MS and electrospray ionization tandem MS (ESI MS/MS) [251, 252]. Coelho et al. [253] applied HPLC HG AFS to the direct determination of As(III), DMA, MMA and As(V) in beer. As(III), DMA, MMA, As(V), AB and AC were determined in fish using HPLC microwave (MW) HG AAS [262]. Gong et al. [263] consider the most useful techniques in the speciation analysis of As in biological samples (including food) to be chromatographic separation techniques coupled with ICP MS, HG AAS and ES MS detection.

Mercury The largest amounts of mercury enter the environment via human agencies. With improvements in trace analysis of this element, it became possible to identify its sources, such as industrial processes and agricultural activities, but also natural volcanic and underwater emissions. The speciation forms of Hg differ in their toxicity, depending on which part of the human body is exposed to them. Organomercury compounds, which are absorbed through the digestive tract, and metallic mercury, which enters the body via the respiratory tract, pose the greatest threat to human health [264–266]. Contact with metallic mercury is rather rare, but contact with organomercury compounds is very common. The main sources of exposure to the latter are seafood and fish [265].

Many human tragedies have been caused by organomercury compounds in food. In the twentieth century, mercury compounds, usually the most toxic types, were applied as fungicides to seeds. Large-scale poisoning occurred in Iraq in 1971–1972 as a result of an appalling error, when seeds contaminated with Hg compounds were sold for consumption. After that event, the application of Hg compounds in farming practice declined, but a fair amount of the element remains in the soil.

The greatest tragedy of all involving mercury unfolded in Minamata Bay, Japan, between 1953 and 1970. Industrial effluents containing mercury compounds were discharged untreated into the bay, and Hg accumulated in fish, which were then caught and eaten by the inhabitants of that coast. Those people soon began to

exhibit symptoms of poisoning, and in the worst cases the result was paralysis, deafness, blindness and even death [265, 267].

The USFDA stipulated a maximum level of contamination of seafood by methylmercury of 1 mg/kg [185]. Consequently, routine speciation analyses had to be implemented in laboratories monitoring the quality of food products before these could be allowed onto the consumer market. The techniques usually used for monitoring food quality are LC coupled with CV AAS or CV ICP MS [172]. The inference to be drawn from van Dael's review [185] is that advanced techniques, such as CV AAS, CV AFS, FT IR and microwave-induced plasma AES (MIP AES), and less sophisticated methods offer comparable selectivity and sensitivity for determining speciation forms of Hg in food to satisfy food quality monitoring requirements.

Taylor et al. [136] performed speciation analysis of Hg in biological samples of marine origin using wet microwave mineralisation in conjunction with GC ICP MS. This method was particularly useful for very small samples. Using CV AAS, Magalhães et al. [268] were able to detect differences in the bioaccumulation by fish of methylmercury and total mercury. Vereda Alonso et al. [120] first applied selective solid-phase extraction and then flow injection (FI) CV AAS and CV GF AAS to determine speciation forms of Hg in seafood. The advantage of this method is that it does not require chromatographic methods but retains high selectivity and sensitivity. Carro and Mejuto [269] reviewed the speciation techniques for determining organic derivatives of Hg in food samples. The following were used to detect methylmercury: GC ICP MS [270], GC electrocapture detection (ECD) [271], HSA GC MIP AES [272], headspace analysis (HSA) GC AFS [273] and GC MIP AES [274]. Pereiro and Diaz [222] described the use of GC MIP atomic emission detection (AED) for the routine quantitative analysis of Hg, Sn and Pb species. Multicapillary GC coupled with ICP MS has also been used in speciation analysis [275]. Grinberg et al. [276] used solid phase microextraction GC furnace atomisation plasma emission spectrometry (SPME GC FAPES) to detect methylmercury and inorganic mercury in fish, achieving limits of detection of 1.5 and 0.3 μg/kg, respectively. Electrothermal vapourisation (ETV) ICP MS enabled the quantitative speciation of Hg without the need for prior sample preparation or risk of the mutual conversion of CH_3Hg and Hg(II) into metallic Hg [277]. Such a conversion was detected during derivatisation preceding GC ICP MS. The technique recommended for the speciation analysis of Hg in fish is ETV ICP MS, and the limits of detection for CH_3Hg and Hg(II) are 2 and 6 μg/kg, respectively [8]. Liang et al. [278] proposed using HPLC CV AFS to determine four speciation forms of mercury (i.e. mercury chloride, methylmercury chloride, ethylmercury chloride and phenylmercury chloride). The method was successfully used to define the level of methylmercury chloride in seafood.

Selenium The toxic properties of selenium are closely linked with its speciation. As a consequence, analytical procedures have been developed enabling Se compounds to be determined in biological and food samples. This element is interesting in that safe levels of its consumption lie within narrow limits: both Se in excess and

Se deficiency lead to serious disease, and its bioavailability is linked with several chemical forms. It has been established that greater amounts of Se accumulate from its organic compounds. In view of this, the determination of the various forms of Se in food is extremely important [8].

Van Dael's review [185] reports on the speciation of Se and the distribution of its various forms in food products such as soya, wheat, enriched garlic, onions, broccoli, Se-enriched yeast, cooked cod, milk (human, cow, goat, sheep). Many of the methods used to determine Se levels are based on GC coupled with AAS, AFS, AES and ICP MS. Moreno et al. [279] used pervaporation (PV) GC AFS to analyse volatile Se compounds in garlic, achieving a range of quantification from 0.45 to 1 mg/kg. An extraction procedure for five speciation forms of Se was devised that required enzymatic digestion (protease XIV, lipase VII, protease VIII) and HPLC microwave-assisted digestion (MAD) HG AFS [197]. With these enzymes it was possible to separate the various forms of Se from the sample, but prior purification of the sample was necessary to enable their analysis using HPLC AFS,. The yields of these various speciation forms were unsatisfactory; however, this may have been a result of their incomplete extraction from mussel tissues [197]. Enzymatic extraction was also applied to cooked cod fillets, in which the content of Se species was determined using anion-exchange chromatography coupled with ICP MS [172]. Kannamkumarath et al. [280] used HPLC ICP MS to determine the distribution and speciation of Se in various kinds of nuts, following prior enzymatic extraction using proteinase K. According to Uden [281], reversed-phase ion pair chromatography and ion chromatography coupled with ICP MS are techniques permitting the separation and quantification of speciation forms of Se in Se-enriched yeast, garlic, mushrooms and seaweed extracts. Reversed-phase ion pair chromatography coupled with ICP MS was also used for the speciation analysis of SeCys, Se-M and Me_3Se^+ in biological samples, including yeast [172]. Yeast was also analysed with reversed-phase chiral chromatography coupled with MW HG ICP MS [172]. In order to determine inorganic forms of Se such as Se(VI) and Se (IV), a method using flow injection (FI) HG AAS was developed [282]. Dernovics et al. [283] put forward an improved procedure for extracting Se from mushrooms using enzymatic processes. According to Stefánek et al. [284], enzymatically treated mushroom samples can be analysed for Se speciation forms using HPLC combined with hydraulic high-pressure nebulisation (HHPN). Analyses of Se compounds have been carried out, for example, in cooked cod fillets [172]; various kinds of nuts [280]; yeasts and nuts [285]; garlic, yeasts, mushrooms and seaweed extracts [281]; selected food products and water [286]; baby food and dietary supplements [287, 288]; and condiments [22]. The review by Guerin et al. [289] describes the usual speciation techniques for analysis of Se and As in food matrices such as fish, mussels, vegetables, mushrooms and mineral water. Substantial progress has recently been achieved using capillary electrophoresis for the speciation analysis of Se in dietary supplements and human milk [290]. The role of Se and the techniques applied in its speciation analysis, for example, ICP MS coupled with ESI MS, ESI MS/MS and matrix-assisted laser desorption/ionisation time-of flight (MALDI TOF), are extensively described by Pedrero and Madrid [175]. HPLC

MS/MS was used by Gosetti et al. [287] to determine Se in dietary supplements. Infante et al. [291] reviewed the analytical procedures applying mass spectrometry to the speciation of Se in food products.

Tin Tin is best known as the metal used to coat cans for food; a thin layer of tin on steel cans prevents the steel from corroding, and, thus, prevents food from going bad. Tin is ideal for this purpose: it is non-toxic, does not corrode and readily forms a coating on other metals. It does not pose any threat to humans, except in the case of a few canned products, usually those with an acidic pH [145, 292–295]. The speciation of tin is important mainly in the context of its organic compounds.

Tributyltin (TBT) is used as an anti-fouling compound to prevent the growth of microbes and fungi, and to prevent the attachment of barnacles and other organisms to ships' hulls. TBT has been found toxic towards aquatic organisms, including fish and marine mammals, rapidly accumulating in them [296].

Because of its great toxicity, TBT has been intensively studied [222, 241, 297–300]. According to Belfroid et al. [301], the mean permissible level of TBT residues in seafood was exceeded in at least one or more samples from 9 out of 22 countries studied(i.e. Canada, France, Italy, Japan, Korea, Poland, Taiwan, Thailand and the USA). GC FPD was used to measure levels of organotin derivatives such as TBT, dibutyltin (DBT9 and monobutyltin (MBT) in marine molluscs [302, 303], fish [302, 304] and fish-eating birds [304]. GC MS selected ion monitoring (SIM) was used to determine organotin compounds in mussels and water [181]. An ion-trap detector (ITD) coupled with GC was used to determine levels of organic derivatives of tin, except MBT [305]. With HPLC HG ICP AES, marine molluscs were analysed for organotin compounds [172]. Marcic et al. [306] employed high-pressure extraction followed by GC pulsed flame photodetector (PFPD) to determine levels of organotin compounds in vegetables. The transformations of phenyltin during the preparation of biological samples for analysis by species-specific isotope dilution (SSID) GC ICP MS were described by Dong et al. [307]. Üveges et al. [308] used GC ICP MS to define the content of butyltin compounds in mussels and sediments. HS stir-bar sorptive extraction (SBSE) and thermal desoption (TD) GC MS were the techniques of choice for the speciation analysis of butyltin and methylmercury in samples of water [309]. Pereiro and Diaz [222] utilised GC MIP AED for the speciation of compounds of Hg, Sn and Pb.

Other Elements The physicochemical forms of an element have a major influence on its bioavailability and the role it plays in the human body. To give an example: chromium can occur in two speciation forms, one of which is biologically active [Cr (III)] and the other [Cr(VI)] toxic. Knowledge of these two forms of Cr is therefore essential from the standpoint of food safety [208]. Kotas and Stasicka [209] provide a detailed description of methods for the speciation analysis of Cr, and for the preparation of samples and their storage. The techniques these authors mention include flow injection analysis (FIA), HPLC (including ion chromatography), ion pair chromatography and ion-pair reversed-phase chromatography coupled with detectors such as UV VIS, GF AAS, ECD, X-ray fluorescence (XRF) and ICP AES.

Ambushe et al. [174] applied dynamic reaction cell (DRC) ICP MS to estimate the content of Cr species in lyophilised samples of milk.

The toxicity of lead varies depending on its forms: organic compounds of Pb are much more toxic than inorganic forms [310]. Barałkiewicz et al. [311] employed HPLC ICP MS and HPLC ESI MS to determine the levels of Cd and Pb species in peas.

Anion exchange chromatography coupled with ICP MS was used in the simultaneous speciation analysis of As, Se, Sb and Te compounds in extracts of fish [230]. Size exclusion chromatography (SEC) coupled with specific detectors is frequently used to analyse species of trace elements in protein-rich materials, such as extracts of meat and plant tissues. For instance, SEC hyphenated with ICP MS was used for the speciation analysis of Cu and Zn in samples of leguminous plants [191]. The same technique was applied to the speciation analysis of Cu, Cd, Zn, Se, As and Ca in fish [220] and Fe, Zn, Cu, Ag, Cd, Sn and Pb in mussels [189]. SEC HPLC coupled with GF AAS turned out to be very useful for determining levels of Fe species in baby food [312]. With gel permeation chromatography (GPC) GF AAS, the speciation forms of Cd were determined in two kinds of vegetables contaminated with this element [216].

9.5 Chemometric Techniques for Evaluating the Results of Trace Analysis

Chemometrics is a branch of science and technology dealing with the extraction of useful information from multidimensional measurement data using statistics and mathematics. It is applied in numerous scientific disciplines, including the analysis of food [313–315]. The most common techniques applied to multidimensional analysis include principal components analysis (PCA), factor analysis (FA), linear discriminant analysis (LDA), canonical discriminant function analysis (DA), cluster analysis (CA) and artificial neurone networks (ANN).

Multidimensional techniques are regularly used in analytical assessments of measurement data relating to the levels of chemical elements in the quality control of animal and plant food products. Chemometric interpretations have been obtained for the following animal products: meat and meat products [316–318], fish [319–321], seafood [25, 322–328], milk and dairy products [329–332] and honey [333–339]. Similar interpretations have been obtained for the following plant products: rice [143], cereals [340], vegetables [140, 341–346], fruit and fruit preserves [347], tea [155, 348–350], coffee [13, 155, 351, 352], mushrooms [26], fruit juices [141], confectionery [21, 353], nuts [354], wine [355–358], beer [66, 359] and other alcoholic beverages [159, 360, 361].

Armed with a knowledge of the mineral composition of animal and plant foods, one can establish their differentiation with respect to geographical provenance, biological genus and species, as well as the degree of processing.

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Chapter 10
Application of Trace Analysis for Medical Diagnosis and Monitoring of Selected Drugs

Wiktoria Struck-Lewicka, Michał J. Markuszewski, Roman Kaliszan, Irena Baranowska, Sylwia Magiera, and Marta Koper

10.1 Metabolomics and Metabonomics

Wiktoria Struck-Lewicka, Michał J. Markuszewski, and Roman Kaliszan

The beginning of the twenty-first century has brought a flourishing of new technologies and innovative diagnostic and analytical tools to face the challenges of current medicine. To cope with these challenges and to understand disease processes there has been development of such studies as genomics, transcriptomics, proteomics, and metabolomics. These and others, the "-omics," create a new type of biology called "systeomics." Moreover, elucidation of the genetic code at the turn of the millennium initiated the development of genomics. However, many questions are still unanswered, especially regarding the relationship between gene expression and pathogenic processes.

Proteins and messenger RNA are directly related to genes (as products of their expression). In turn, metabolites can be treated as intermediates products of the genetic code. For this reason, analysis of the genome is difficult to associate with analysis of metabolites. Moreover, metabolomics seems to be more difficult to analyze and biologically interpret than other -omics. However, this does not mean that metabolomics cannot offer invaluable information. On the contrary, this

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domain complements knowledge about the observed relationships between the genome and pathogenic processes.

The body comprises 30,000–50,000 genes, 150,000–300,000 transcriptomes, and approximately a million proteins, but the amount of cellular metabolites (endogenous low-molecular-weight compounds) is in the range of 3500 to 10,000. Compared with the number of transcriptomes and proteomes, the number of metabolites is relatively small, but the number of dependencies that are included in the final metabolic profile is multidimensional. Such dependencies, together with number of metabolites, make metabolomics both an interesting and complicated domain. In addition, the development of both analytical techniques and advanced bioinformatics methods has meant that potentially important metabolites, hitherto impossible to detect because of their trace amounts, have been successfully quantitatively analyzed and interpreted. The obtained metabolomics data sets, after bioinformatics evaluation, complement information about the human body condition, and, together with proteomics and transcriptomics, provide a holistic view of biological processes and the relationships between them.

10.2 Metabolomics in Systems Biology

The origin of metabolomics, as a part of systems biology, can be dated to the 1970s when Arthur Robinson and coworkers, in research regarding the nutritional needs of volunteers, proposed taking into account and analyzing not only one but many of the chromatographic peaks derived from metabolites in their urine [1].

According to Nicholson et al. [2], metabolomics and metabonomics specify, in a comprehensive manner, the biochemical profile and function of sets of metabolites by analysis of the metabolic composition of single cells, biological fluids, or tissues. In the literature, however, definitions of metabonomics and metabolomics are unequivocal. *Metabonomics* concerns study of metabolic profiles at the level of the whole organism by analysis of biological fluids such as blood, urine, and cerebrospinal fluid. *Metabolomics* concerns simultaneous quantitative and qualitative determination of as many cellular metabolites as possible. Additionally, by analogy with the genome, transcriptome, and proteome, the concept of the *metabolome* was created and defined as the total number of low-molecular-weight compounds (metabolites) in a given cell. Moreover, Nicholson and Wilson [3] provided *metabonome* as an additional term, defined as the sum of all cellular metabolites and products of their interactions in the organism.

Comprehensive quantitative and qualitative analysis of the metabolome is essential for full understanding of cellular functions. It was noted that although small changes in the concentration of an enzyme slightly affect the overall variability in a biochemical pathway, the levels of cellular metabolites can be significantly altered. The metabolome, as a derivative of the proteome, is directly related to changes occurring in the proteome; therefore, the levels of metabolites reflect these changes at an amplified scale as the end products of interaction between

genome, transcriptome, and proteome. Therefore, one can say that analysis of the metabolome can be treated as a more sensitive tool than analysis of high-molecular-weight compounds from other -omics. Additionally, study of the metabolome enables easier understanding of complex biological systems and changes occurring during disease and after applied pharmacotherapy.

Each -omic creates a common base of systems biology. There are, however, significant differences between metabolomics and genomics, transcriptomics and proteomics. Two aspects of these differences can be considered, namely, theoretical and technical. From a theoretical point of view, there are no simple relationships between genes and metabolites as there are between genes, messenger RNA molecules, and proteins. A single gene can determine the presence of a specific metabolite in a given cell but might not influence its level. This depends on the enzyme activity in a given metabolic pathway as well as on effectors that act on each enzyme. From a practical point of view, the levels of metabolites vary depending on different extra- and intrapersonal factors, such as the physiological and pathological state of the body.

With respect to technical differences, it should be emphasized that, for metabolomic analyses, there is no single analytical technique that allows determination of all low-molecular-weight compounds, unlike genomic and proteomic analyses. In metabolomics, various complementary analytical techniques are often used in combination with advanced bioinformatics tools for analysis of large data sets.

The size of the metabolome varies and depends on the species and type of organism. In the case of unicellular organisms such as baker's yeast (*Saccharomyces cerevisiae*), the number of metabolites amounts to 600, which is only a tenth of the number of genes (6000). In turn, plants are characterized by a much larger number of metabolites. The metabolome of Arabidopsis (*Arabidopsis* sp.) is estimated to be 5000 compounds. In total, the number of metabolites of all plant species is estimated at 90,000–200,000 compounds, of which secondary metabolites make up a large proportion.

The number of human metabolites has been estimated differently by various scientists. For example, according to Kell [4], the size of the human metabolome is calculated at approximately 2700 metabolites, which are involved in 1100–3300 reactions. This is only an estimated value because the human metabolome has not been thoroughly calculated and substrates for many enzymes have not yet been discovered. Additionally, difficulties in estimation of the appropriate size of the metabolome can be attributed to the fact that many determined metabolites are exogenous in origin (derived from diet or drugs) or derived from endogenous bacterial flora. So far, the online *Human Metabolome Database* (http://www.hmdb.ca) contains more than 4500 metabolites detected in human blood and 3995 metabolites detected in human urine. This database is constantly expanded and updated. It is estimated that it will eventually contain more than 10,000 metabolites.

10.3 Metabolomics Tools

With the advent of modern analytical techniques, together with advanced preprocessing methods, it became possible to determine large numbers of metabolites in a relatively short time with good reliability. Previously applied biochemical assays were limited to determination of a single, earlier identified metabolite or known group of metabolites. Analytical progress allowed the use of such advanced technologies as nuclear magnetic resonance (NMR) and mass spectrometry (MS), which, in turn, entailed the use of multivariate mathematical approaches for calculation of the large data sets obtained after analysis.

Because of large variations in the chemical structures of metabolites, it is practically impossible to find a single analytical technique that can determine all metabolites present in biological samples. Many metabolites are polar, nonvolatile, and do not have chromophore groups in their structure, which makes them difficult to detect. Hence, the modern comprehensive analytical approach in metabolomics is based on use of various complementary analytical techniques to enable large numbers of metabolites to be detected and determined.

A typical metabolomics workflow consist of three main phases: sample pretreatment, analytical determination, and bioinformatics data calculation.

10.3.1 Sample Pretreatment

Sample pretreatment is an extremely important step in metabolomic studies as it has a great impact on the final results. Sometimes this step can be omitted or significantly reduced, depending on both the strategy and analytical technique used. In the case of untargeted study such as metabolite fingerprinting, wherein as many metabolites as possible are determined, sample pretreatment is limited to deproteinization (for blood), centrifugation, and filtration steps. Sample pretreatment is also reduced when NMR is used, as the sample can be diluted in deuterated water (D_2O). NMR is practically nondestructive, which allow reuse of the same sample for subsequent analysis. This is an exception among analytical techniques, because usually the sample undergoes irreversible changes during analysis and cannot be reused for another determination.

Liquid–liquid extraction (LLE), solid phase extraction (SPE), and solid phase micro-extraction (SPME) are frequently applied for sample pretreatment. The type of extraction is highly related to the type of metabolites selected for determination. In previous metabolomics studies, extraction was focused on compounds of adequate stability that could be extracted together (carbohydrates, esters, amino acids, or organic acids).

Metabolites are mostly extracted into aqueous or methanolic solutions. Subsequently, they are separated and analyzed as separate fractions, either aqueous or lipophilic. Here again, it should be emphasized that there is no single extraction

method that is adequate for all metabolites. The applied conditions suitable for extraction of one group of metabolites might cause degradation of another group with different physicochemical properties. For instance, extraction of alkaloids is performed under basic conditions, which are unfavorable for separation of aldehyde compounds. Similarly, in the case of SPE, the selection of sorbent bed determines the selectivity for given metabolites. Application of cation-exchange sorbent beds can be suitable for extraction of anionic compounds, whereas anion-exchange sorbent beds are selective for cationic metabolites. There are also other sorbent beds that are highly selective for a characteristic chemical group in the chemical structure of extracted compounds. For instance, nucleosides undergo extraction using a phenylboronic acid sorbent bed that is characterized by high affinity to 1,2- and 1,3-*cis*-diol groups existing in structure of nucleosides. However, such extraction would not give satisfactory results for deoxynucleosides as these compounds do not have *cis*-diol groups in their structure.

Moreover, in terms of the large range of properties of analyzed metabolites, the extraction method is practically useless. Often, it can be replaced or preceded by a derivatization step, as is commonly performed for thiol compounds. These compounds are very unstable but application of an adequate derivatization step converts them into stable derivatives, allowing their determination. Unfortunately, in such a situation, many relevant metabolites are neglected because further analysis is focused only on those that have been subjected to chemical modification.

10.3.2 Analytical Determination

NMR is a technique that uses a magnetic field to determine the structural composition of organic compounds. This technique can be used for determination of large numbers of chemically different cellular metabolites, and for their structural identification. However, this technique is not sensitive enough for analysis of trace amounts of metabolites. The information obtained after NMR analysis is described as the NMR spectrum, wherein each range is assigned to the exact structure of an analyte. Such spectra are often regarded as molecular fingerprints.

A much more sensitive technique than NMR is MS detection combined with various separation techniques (e.g., high-performance liquid chromatography, HPLC; ultrahigh-performance liquid chromatography, UHPLC; capillary electrophoresis, CE; gas chromatography, GC) that allow previous separation of metabolites. The application of MS enables analysis of large number of metabolites based on their interactions with the separation system and, above all, on the basis of their molecular weights.

Gas chromatography coupled with mass spectrometry (GC-MS) commonly requires a derivatization step in order to enhance both the stability and amount of determined metabolites. The separated metabolites, after derivatization, are introduced to the mass spectrometer, wherein they undergo ionization and are selectively determined according to their mass-to-charge ratio (m/z). Using GC-MS,

Fiehn et al. [5] were able to determine 326 metabolites in *Echinacea* plant extracts. Only half of them have been identified. The identification of the rest should be possible by applying GC coupled with tandem MS (GC-MS/MS) or NMR.

The application of two-dimensional gas chromatography (GC/GC) coupled with MS detection is helpful in separation and identification of metabolites that cannot be separated with the GC-MS technique. The advantage of two-dimensional GC/GC is that it combines chromatographic columns of different polarity and enables analysis at different temperatures. As a result, previously unresolved metabolites can be separated and determined.

In contrast to GC, liquid chromatography hyphenated with mass spectrometry (LC-MS) does not require a derivatization step before sample analysis. Separation of metabolite from sample matrix is achieved using chromatography columns with various stationary phases of different physicochemical characteristics. LC-MS is more often used than GC-MS because it is more suitable for unstable compounds, compounds difficult to derivatize, and nonvolatile compounds [6, 7]. Therefore, a wider range of metabolites with various physicochemical properties can be determined using LC-MS. Moreover, the sample pretreatment procedure is much simpler, which can have a great impact on minimization of analytical variability.

Capillary electrophoresis coupled with mass spectrometry (CE-MS) is relatively rarely used in comparison with LC-MS or GC-MS. However, the main advantages of CE are high separation efficiency, which enables between 100,000 and 1,000,000 theoretical plates to be obtained in a relatively short time (~10 min); low volumes of analyzed samples (nanoliters) and buffers (milliliters); and wide range of applicability, from low molecular weight compounds to biopolymers. The low consumption of expensive and harmful chemicals gives the added advantages of low cost and being in line with green chemistry principles. Unfortunately, one of the limitations of CE is its sensitivity; this is relatively low when calculated as molar concentration (concentration sensitivity) but high when calculated as molecular weight (mass sensitivity). This fact is particularly important in determination of metabolites present in trace amounts. Moreover, compared with LC, the repeatability of analyte migration/retention time is slightly worse, which is explained by less stable electro-osmotic flow in CE [8].

Fourier-transform infrared spectroscopy (FTIR) is based on the measurement of absorbed light in the infrared range by the sample being analyzed. From the obtained spectra, it is possible to identify specific functional groups and structures. In metabolomics studies, FTIR is used for determination of complex mixtures and can be combined with LC and GC techniques [9, 10].

10.3.3 Bioinformatic Analysis of Obtained Data Sets

The development of analytical techniques involves implementation of new and advanced computational methods that enable multivariate data preprocessing and their exploration. The metabolic profiles, previously determined using an adequate

separation technique, can be subsequently analyzed with the use of chemometric methods. Raw data sets, however, can contain many distortions caused by analytical variability (background noise, shifts in analyte migration/retention times, baseline drift, or reduction in sensitivity over time). The application of adequate chemometric preprocessing methods enables unwanted drifts or signals from raw data sets to be eliminated without risk of losing important information. It should be mentioned that chemometric methods are commonly used at each stage of the data pretreatment procedure: from de-noising and baseline correction (Savitzky–Golay and Cooley–Tukey algorithms, Fourier transformation) to synchronization of shifted signals and analyte retention/migration times (correlation optimized warping, dynamic time warping, or parametric time warping) [11]. Often, application of chemometric preprocessing methods for the obtained data sets allows exploration and elucidation of the relationships between variables using principal component analysis, cluster analysis, or discriminant analysis.

Principal component analysis enables reduction of a large data matrix into two or three main components that include orthogonally relevant information. In such a way, changes in metabolic profiles, described by many variables, can be measurably determined and compared. Subsequently, using other calculation procedures for the reduced data matrix, the importance of variables (metabolites) can be determined and assessed. Discrimination or regression calculation methods are of great importance in this step of the analysis.

Another important aspect of metabolomic data analysis is visualization of the results. After application of effective bioinformatics tools for data visualization, the multivariate data matrices can be more easily compared.

10.4 Research Strategies in Metabolomic Studies

In metabolomic studies, there are three commonly used research strategies, namely, targeted metabolomics, metabolite profiling, and analysis of metabolic fingerprints [12].

1. Targeted metabolomics is used for analysis of known, previously identified metabolites, for instance, markers of a changed biochemical pathway or gene mutation. The selected metabolite, which can be a substrate or product of a changed pathological process, is quantitatively determined using calibration curves and stable isotope-labeled internal standards. The measured levels can therefore be treated as indicators of a changed pathological process in the body. Because the metabolites are quantitatively determined, their selective extraction is crucial in the sample pretreatment procedure. Therefore, application of adequate, validated sample pretreatment steps can significantly enhance the sensitivity of analysis, which is especially important for determination of metabolites in trace amounts (phytohormones).

2. Metabolite profiling is based on analysis of a group of metabolites or certain range of metabolites as a quantitative or screening method. This strategy is often used for analysis of metabolites from a specific biochemical pathway (Krebs cycle, glycolysis) or metabolites with common physicochemical properties (carbohydrates, amino acids). Similarly to the targeted approach, sample pretreatment is also selective for the desired metabolites. Therefore, the aim is to reduce the effect of the sample matrix as much as possible.
3. Metabolite fingerprinting is widely used in functional genomics and clinical diagnostics. This strategy is focused on analysis of all metabolites present in biological samples, determined using various, complementary analytical techniques. This approach is an example of screening analysis, wherein samples are qualitatively analyzed and compared with each other. As a result of analytical determination, the obtained spectra are compared using multivariate chemometric approaches in order to find metabolites that discriminate analyzed samples by their biological origin or state of the body. Limitations of this approach are the low reproducibility of analytical methods and the effect of the sample matrix, which can sometimes cause problems with correct sample classification and in interpretation of results.

10.5 Examples of Application of Metabolomics in Analysis of Low Amounts of Metabolites

10.5.1 Electrophoretic Analysis of Metabolic Profiles of Glycolysis Cycle Metabolites in Human Erythrocytes

In humans, erythrocytes are a specific type of cell that do not contain organelles such as nucleus and mitochondria. The energy required for proper functioning of erythrocytes comes almost entirely from the glycolysis cycle. Metabolites from the glycolysis cycle are routinely measured by enzymatic methods. However, the limitation of an enzymatic approach is that only one metabolite can be determined during a single analysis. Application of CE with indirect spectrophotometric detection is an alternative to enzymatic methods and also to methods involving capillary electrophoresis coupled with mass spectrometry (CE-ESI/MS) [14]. Although CE with indirect spectrophotometric detection is less selective and sensitive than CE in combination with MS, it has advantages such as low complexity of equipment, ease of analysis, and relatively small effect of interfering background components [15–17]. The metabolites of glycolysis cycle, such as glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (F1,6P), dihydroxyacetone phosphate (DAP), 2,3-diphosphoglycerinic acid (2,3-DPG), 3-phosphoglyceric acid (3PG), phosphoenolpyruvate (PEP), pyruvic acid (PYR), and lactic acid (LA), are low molecular weight compounds with no strong chromophore groups

in their structure. Therefore, methods using indirect spectrophotometric detection seem to be suitable for determination of these metabolites. After optimization of the method [13], the following electrophoretic separation conditions were used: background electrolyte consisted of 20 mM 2,6-pyridinedicarboxylate and 4 mM cetyltrimethylammonium bromide (CTAB) at pH 12.3; the unfused silica capillary had a total length of 100 cm (90 cm to detector) and an internal diameter of 50 μm; applied voltage was 25 kV; capillary temperature was controlled at 15 °C; and sample was loaded using hydrodynamic injection for 20 s.

The inter-day and intra-day repeatability of the method, expressed as coefficient of variation (CV %) for migration times of determined metabolites, ranged from 0.3 to 1.9 % and from 3.8 to 5.1 %, respectively. The inter-day and intra-day repeatability for peak area ranged from 2 to 10.4 % and from 10.2 to 15.8 %, respectively. The linearity of the method was 12.5–2000 μM and was characterized by regression equations with high regression coefficients of 0.997–0.999 (with the exception of 2,3-DPG, for which the regression coefficient was 0.991). The limit of quantification of analyzed metabolites were set from 38.1 μg/mL (5×10^{-5} M) for 2,3-DPG to 1.29 μg/mL (6.25×10^{-6} M) for PEP.

The validated method was subsequently applied for determination of metabolic profiles of metabolites involved in the glycolysis cycle from blood samples derived from 22 healthy volunteers. In order to isolate erythrocytes, the samples were centrifuged, followed by lysis and ultrafiltration. The determined mean concentrations of metabolites in erythrocytes ranged from 49.6 ± 23 μM for F6P to 3.1 ± 0.9 mM for 2,3-DPG.

The obtained results were subsequently compared with those available in the literature, but achieved using immunoassay methods [18–20]. For most of the determined metabolites of the glycolysis cycle, the mean concentrations agreed with those found in the literature. The exception was DAP, with a mean concentration twice that obtained by Minakami and Yoshikawa [18]. However, the results of DAP determination using immunoassay varied between each study, ranging from 138 μM [18] to 9 μM [20]. Of the many additional peaks observed in electropherograms (apart from those resulting from metabolites of the glycolysis cycle), one peak was identified as corresponding to inorganic phosphate. Moreover, the highest concentration was measured for 2,3-DPG, which is a specific metabolite of the erythrocyte glycolysis cycle. 2,3-DPG plays an important role in hemoglobin affinity to oxygen and facilitates the release of oxygen from oxyhemoglobin. The obtained results confirm the high specificity and sensitivity of the developed method. The method is relevant from a biological point of view because it allows determination of all seven major metabolites involved in the glycolysis cycle in single run lasting less than 15 min. This is the major advantage over enzymatic methods, which allow determination of only one metabolite per run. Moreover, the developed method seems to be suitable for comparative study of the metabolic profiles of glycolysis cycle metabolites in patients and healthy volunteers.

10.5.2 Determination of Metabolic Profiles in Urine of Patients with Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is currently the sixth most common cancer and the third leading cause of cancer death worldwide. The prognosis for HCC depends mainly on the stage of the disease. In addition, the currently used serologic test using α-fetoprotein (AFP) is not considered to be sufficiently powerful because of high false-positive and false-negative results. Therefore, there is a need for new biomarkers of HCC that are specific and sensitive enough to detect HCC using biological samples.

The study was performed employing GC-MS, using bis-trimethylsilyl-trifluoroacetamide (BSTFA) as derivatization agent during sample preparation. The aim of the study was to compare the metabolic profiles of urine samples from patients with liver cancer ($n = 20$) and healthy volunteers ($n = 20$) and, subsequently, to develop a diagnostic model that would include selected metabolites of potential diagnostic significance in HCC [21].

Sample pretreatment is essential for obtaining satisfactory results. In order to selectively determine metabolites from a biological matrix using GC-MS, a derivatization step is necessary. For this purpose, urine samples were incubated at 37 °C (3 min) and centrifuged (15 s). To 1 mL of supernatant, 800 μL of methanol and 100 μL of L-2-chlorophenylalanine were added. The obtained mixture was centrifuged (5 min) and sonicated. Subsequently, the pH of samples was adjusted to 9–10 using 0.5 M NaOH. Samples were then filtered through membrane filters of 0.45 μm internal diameter and 100 μL aliquots of filtrate evaporated to dryness under nitrogen stream. These samples underwent derivatization with the use of 100 μL BSTFA and 1 % TMCS (trimethylchlorosilane) at 100 °C for 1 h [21].

After optimization of the method, the following analytical conditions were used: silica capillary with stationary phase HP-5MS (30 m × 0.25 mm internal diameter, 0.25 μm film thickness); column gradient temperature started at 80 °C and increased at a rate of 10 °C/min to 280 °C; injection temperature was fixed at 250 °C; and helium was used as a carrier gas with constant flow rate of 1 mL/min. A mass spectrometry detector with single quadrupole was used as analyzer, operated at 150 °C with an acquisition scan rate of 50–800 m/z.

As a result of GC-MS analyses, 103 metabolites were determined, of which 66 were successfully identified and 18 were used to create a diagnostic model. Of these 18 metabolites, 5 (suberic acid, glycine, L-tyrosine, L-threonine, and succinic acid) had significantly higher levels in patients with HCC than in healthy volunteers ($p < 0.05$). Other metabolites (oxalic acid, xylitol, urea, phosphates, propanoic acid, threonine, pimelic acid, butyric acid, trihydroxypentanoic acid, hypoxanthine, arabinofuranose, dipeptide of hydroxyproline, and tetrahydroxypentanoic acid) showed higher levels in healthy volunteers ($p < 0.05$). In addition, Wu et al. determined the levels of AFP using an ELISA test in serum from the same patients and healthy volunteers as in the metabolomic study of urine samples. An AFP concentration above 20 ng/mL suggests a positive result and the presence of

HCC. In the studied group, 75 % of patients were correctly classified into the HCC group using AFP marker, whereas healthy volunteers were negatively diagnosed with HCC. The results of the study using the AFP markers show that AFP is not suitable as a universal marker, because 25 % of patients known to suffer from liver cancer did not show positive results. The average sensitivity and specificity of this marker are 40–65 % and 76–96 %, respectively.

The authors also combined metabolomics results with results obtained from AFP determinations. The model was created using linear discriminant analysis. Principal component analysis was also carried out. Thanks to the created model, it was possible to detect metabolites of potential diagnostic value. Moreover, analysis of metabolomic profiles decreased the number of patients that were incorrectly classified with the use of AFP marker [21].

10.5.3 Metabolomic Analysis of Plasma Samples from Patients with Amyotrophic Lateral Sclerosis Using NMR

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease that leads to degeneration of corticospinal tract, spinal anterior horn neurons, and brainstem. ALS is extremely difficult to diagnose because of the diversity of symptoms and their gradual progression. Therefore, diagnosis of a patient can last from a few months to a year. The average survival is 2–4 years. During disease development, patients gradually suffer from systematic mobility deterioration as a result of muscle wastage. At later stages, patients have difficulties in speaking, swallowing, and breathing. Patients usually die from respiratory failure. The etiology of the disease is not well understood but current hypotheses involve genetic mutation, protein aggregation, glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction, and microglial activation.

Kumar et al. [22] examined plasma of 30 patients suffering from ALS and 10 patients with Hirayama disease (focal motor neuron disease that resembles ALS, affecting upper limbs), and 25 healthy volunteers. The determination was performed with the use of proton nuclear magnetic resonance (HNMR). Analytes were determined in the range 2.0–2.2 ppm. In the case of non-definite confirmation of metabolite identity, the authors additionally applied two-dimensional double quantum filtered correlation spectroscopy (DQF-COSY), as well as total correlation spectroscopy (TOCSY). Statistical analysis was performed using the U Mann–Whitney test. Mean significant differences in more than two groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test. The correlation between duration of disease and metabolite concentration was calculated using Spearman's correlation test.

As a result of statistical analysis, the levels of glutamate ($p < 0.001$), β-hydroxybutyrate ($p < 0.001$), acetate ($p < 0.01$), acetone ($p < 0.05$), and formate

($p < 0.001$) were significantly higher in ALS patients than in healthy volunteers. In turn, levels of glutamine ($p < 0.02$) and histidine ($p < 0.001$) were significantly lower in ALS patients than in healthy controls. Moreover, the results indicated that concentrations of such metabolites as alanine, lysine, pyruvate, citrate, glucose, creatine/creatinine, and tyrosine were comparable in the ALS group and in healthy volunteers. In the case of patients with Hirayama disease, concentrations of glutamate ($p < 0.01$), pyruvate ($p < 0.05$), and formate ($p < 0.05$) were significantly higher than in the control group [22].

Moreover, the authors examined possible correlations between levels of metabolites and the duration of ALS. As a result, the concentrations of two metabolites with different levels in ALS patients and healthy subjects showed significant correlation with ALS duration. Glutamate showed a positive correlation between disease duration and metabolite concentration ($p < 0.001$, $r = 0.6487$), whereas histidine showed a negative correlation ($p < 0.001$, $r = -0.5641$) [22].

Data analysis also revealed that glutamate concentration was significantly higher in the plasma of patients with ALS and patients with Hirayama disease. Glutamate is one of the most important neurotransmitters, controlling the process of growth and migration of immature nerve cells. This metabolite is also thought to activate excitotoxicity, which leads to the process of neuronal death observed in ALS disease. Furthermore, the decreased level of glutamine observed in patients with ALS could represent an imbalance in the glutamate–glutamine conversion cycle that occurs in postsynaptic buttons and astrocytes during excitotoxicity.

The level of another metabolite, formate, was also elevated in ALS and Hirayama patients. This metabolite is responsible for disruption of mitochondrial electron transport and energy production by inhibiting cytochrome oxidase activity. Cell death as a result of formate inhibition of cytochrome oxidase is believed to be the result of partial loss of ATP and, therefore, the loss of energy needed for basic cell functions. In addition, inhibition of cytochrome oxidase by formate can facilitate the production of cytotoxic reactive oxygen species, which also implies cell death.

A third metabolite, histidine, with decreased levels in ALS patients compared with healthy volunteers, is considered to be an antioxidant. Therefore, the lower levels observed in ALS patients might cause stronger attack of oxygen-free radicals. Furthermore, it was reported that low concentrations of histidine are associated with energy loss and inflammation.

The presented study of metabolites in plasma samples from patients and healthy controls indicates how changes in the concentration of individual metabolites can reflect processes that occur in the body. ALS and Hirayama disease are a significant problem for diagnosis. Therefore, the proposed selected metabolites could be useful as potential markers in diagnosis of these diseases.

10.5.4 Determination of Urinary Nucleosides as Potential Cancer Markers Using High Performance Liquid Chromatography with Tandem Mass Spectrometry

Nucleosides are metabolites of ribonucleic acid (RNA). As a result of excessive turnover of RNA, which occurs in such pathological conditions as cancer, inflammation, and AIDS, nucleosides are excreted into urine in increased amounts. Normal nucleosides (adenosine, guanosine, cytidine, uridine) either undergo further degradation into uric acid, β-alanine, or β-aminoisobutyrate or are reutilized. On the other hand, modified nucleosides, especially methylated nucleosides, are excreted unchanged in urine. Therefore, it seems that elevated levels of modified nucleosides could be an indicator of the presence of cancer and used as a diagnostic tool in cancer prognosis [23].

Struck et al. [23] determined nucleoside levels in urine of healthy subjects ($n = 61$) and patients suffering from urogenital track cancer ($n = 68$) using HPLC-ESI-MS/MS. The sample pretreatment procedure was based on SPE with phenylboronic acid sorbent beds. As a result of method optimization, the chromatography conditions were set as follows: Zorbax Extend-C18 column (2.1 mm × 50 mm, 1.8 μm) with low dispersion in-line filter with 2 μm frits; two mobile phases in gradient elution such that mobile phase A consisted of an aqueous solution of 0.05 % formic acid and mobile phase B consisted of 0.05 % formic acid in methanol; and flow rate of 0.3 mL/min. The mass spectrometric parameters were optimized regarding fragmentor voltage, capillary voltage, and collision energy voltage for each metabolite; ion source parameters were also optimized.

As a result of chromatographic determination, the authors successfully quantified 12 nucleosides, of which five (N_2,N_2-dimethylguanosine, inosine, 3-methyluridine, 6-methyladenosine, and N_2-methylguanosine) revealed statistically significant differences in concentration between the studied groups. This was confirmed by statistical analysis using the U Mann–Whitney test ($p < 0.05$). For data that contained statistically significant nucleosides, discrimination unsupervised multivariate statistical analyses were applied, such as K-nearest neighbors (K-NN) and partial least squares discriminant analysis (PLS-DA). As a result, the sensitivity and specificity of the model consisting of five statistically significant metabolites was calculated. The sensitivity and specificity for the K-NN model were 71.43 and 50 %, respectively, whereas for PLS-DA model they were 42.86 and 88.89 %, respectively.

Other publications also confirm the potentially significant role of nucleosides in the diagnosis of urogenital tract cancer [24, 25]. The use of sensitive determination techniques (e.g., LC-MS/MS) for analysis of metabolites from biological samples of healthy subjects and cancer patients allows detection of significant differences between even small concentrations of metabolites (as can occur in the case of nucleosides) and selection of potential cancer markers that could be used to diagnose the disease at an early stage of development.

10.6 Conclusions

The metabolomic approach uses both sensitive and selective analytical techniques, as well as multivariate chemometrics methods, which make it a potentially useful tool for determination of trace amounts of metabolites in biological and environmental samples. Application of the appropriate metabolomic approach (e.g., targeted or untargeted metabolomics) determines the method of analysis of metabolites (quantitative or qualitative measurements). In quantitative measurements, even small changes in the concentrations of metabolites can be determined, which enables assessment of exact differences between studied groups, such as healthy controls versus diseased patients. On the other hand, in a qualitative approach, a large set of metabolites at different concentration levels is obtained, which needs application of proper data pretreatment to extract information on given metabolites from the multidimensional data sets. The assessment of selected metabolites at various levels from both approaches (even at trace amounts) can be carried out statistically to evaluate their sensitivity and specificity in terms of prognostic and diagnostic value for a particular disease. The information can also be successfully used to help understand some pathological processes occurring in the body at molecular level.

10.7 Determination of Selected Drugs and Their Metabolites in Biological Fluids

Irena Baranowska, Sylwia Magiera, and Marta Koper

10.7.1 Introduction

Recent years have witnessed an increased interest in research that enables better understanding of pathological phenomena responsible for the creation and progression of various diseases. Thanks to new diagnostic tools, it is currently possible to recognize the basis for the most complex anomalies. Great progress in the treatment of many diseases has been noticed, the result of which is extension of patient's lives.

Significant progress has been also made in the diagnosis and pharmacological treatment of many diseases. We are witnessing the emergence of a new generation of drugs, and all pharmacological actions affect the procedures aimed at improving treatment results. Drug concentration measurement conducted during therapy is appropriate when there is a correlation between concentration of drug in the blood and its reaction, and when the pharmacokinetics of a drug are complex and vary with individual patients as a result of differences in both metabolism and other

preparations being taken. Increasingly, drug therapy of diseases is comprehensive and individually tailored to each patient; however, mutual interaction of drugs taken by patients with other substances that affect the final result of their use is one of the most important problems of modern medicine.

Diverse metabolism resulting from genetic polymorphism affects an individual's response to medications applied in the treatment of various diseases, the method and progress of treatment, and the probability of occurrence of favorable or adverse effects. The possibility of determining drug metabolites allows specification of an individual dose of drug and choice of an alternative drug in the event of metabolic disturbances. In addition to searching for abnormalities in human metabolic pathways, which are reflected in pathological states of biological systems, the second major area of interest for clinicians is the search for disease markers and the ability to monitor their concentrations in biological fluids.

Correlation of the concentration level of these metabolites with the stage of a disease could make it easier to take decisions regarding treatment. Because cellular changes always preempt clinical symptoms, finding irregularities in metabolite concentrations before any symptoms of diseases appear, thanks to early implementation of treatment, could slow or inhibit progression of the disease.

10.7.2 Multivariable Diagnostics

Multivariable diagnostics (disease markers, metabolites of used drugs and endogenous compounds, metabolites of dietary components such as antioxidants) can greatly help in improving the design of clinical trials and early monitoring of the effects of drugs.

Linking these data with a concrete therapy is important in clinical practice, in the development of new drugs, and in diagnosis. Moreover, it enables, to some extent, the personalization of effective treatment methods, and not only treatment based on standards. For this reason, a project involving drug monitoring during therapy has been created, "therapeutic drug monitoring" (TDM), based on the collaboration of several scientific disciplines, in particular pharmacokinetics, pharmacodynamics, and analytical chemistry [26]. TDM consists in determination of drug concentrations in body fluids to help develop the most effective, but also the maximum, safe drug dosing regimen for an individual patient. Determination of pharmacodynamic and pharmacokinetic parameters of drugs at the design and study stage, before release of preparations for general use, allows prediction of their distribution and metabolism in an organism. However, the individual characteristics of a patient and the wide use of multiple drugs at the same time (possibly resulting in mutual interactions) have forced the need for control of drug concentrations during therapy.

Patients hospitalized in intensive care units (postoperative) often require the application of combination treatments (i.e., simultaneous use of different drugs). This situation is caused by the necessity of simultaneous control of complex clinical

problems in patients with multiple organ dysfunction. Also, during the course of chronic disease treatment requiring continuous application of basic therapy, exposure to viral or bacterial infections, injury, etc. is possible. In these situations, additional symptomatic treatment is required (e.g., antibiotic therapy, antipyretic and analgesic treatment). In cases of combination treatment, it is necessary to know the possibilities of interaction between drugs, mechanisms of their action, and possible effects on the pharmacokinetics of the drugs [27]. Polypharmacy phenomena can also occur if a patient, as a result of the large availability of pharmaceutical drugs, takes other medication without a prescription. Interactions between medications can cause an increase in toxicity and severe therapeutic complications.

10.7.3 Role of Bioanalysis in Personalization of Therapy

Therapeutic drug monitoring in body fluids is one of the areas of interest of clinical chemistry. Monitored therapy is an optimum method of drug treatment, especially for newly developed drugs whose characteristics, pharmacokinetics, and bioavailability are not entirely known. Monitoring the concentrations of a drug and its metabolites in biological fluids aims to determine and eliminate the adverse effects of therapy. In addition, monitoring enables tracking the progress of treatment and customization of dosage to allow for interindividual differences in drug metabolism. Interindividual differences in drug response and metabolism are the result of mutual interactions between environmental factors (diet, disease, other drugs, exposure to other xenobiotics), genetic factors (genotype, sex, and race), and bioavailability, which is individually very diverse [28].

The concept of personalized medicine is based on understanding the differences between patients suffering from the same illness, and learning about the complex mechanisms of various diseases. Personalized treatment should adapt the treatment to the molecular subtype of disease and consider individual variability in drug metabolism (pharmacogenomics). In other words, treatment should involve the appropriate drug at the right time and in the appropriate dosage. Making therapeutic decisions based on the individual patient's biological characteristics increases the effectiveness of therapy and minimizes the risk of toxicity and undesirable effects. Consequently, through individualization of the diagnostic–therapeutic process, molecular medicine creates opportunities for new, more efficient health care, in relation to both individual patients and the population-based health care system. Hippocrates stated, 1000 years ago, that "It is far more important to know what person the disease has than what disease the person has" [29].

10.7.4 Pharmacokinetics and Drug Bioavailability

An intensely developed area of research is bioanalytical study of the pharmacokinetics and bioavailability of drugs. Pharmacokinetics is study of the biochemical and physiological effects of drugs on the body, and mainly involves quantitative and mathematical evaluation of the processes of absorption, distribution, biotransformation (metabolism), and excretion. Pharmacokinetics based on mathematical analysis of measurements of drug concentrations in the blood or urine allows determination of the pharmacokinetic parameters to be used in a rational and safe pharmacotherapy. A pharmacokinetics study is usually carried out by determination of total drug concentration in a biological matrix (blood, serum, plasma, urine, or saliva) or of metabolites produced in the body at specific intervals. Based on changes in the concentration of the tested component (or group of components), it is possible to determine a mathematical relation between the dose given and those concentrations and to calculate appropriate parameters that qualitatively determine the pharmacokinetic properties of the tested drug.

The most important pharmacokinetic parameters include bioavailability, volume of distribution, clearance, and biological drug half-life. Bioavailability is a parameter that determines the rate and amount of drug absorbed into the bloodstream from the place of its administration, and thus determines its concentration at the site of action and therapeutic efficacy. Bioavailability is defined as the fraction (percentage) of drug dose that passes into the systemic circulation following extravascular drug administration. The degree and rate at which the drug enters the bloodstream are characterized by the following parameters: area under the plasma concentration curve for the drug in the blood (AUC), maximum concentration of drug in the blood (C_{max}), and (if available) time at which the maximum concentration is reached (T_{max}). In order to determine the bioavailability of a given dosage form, it is necessary to determine all three parameters. AUC measurement enables calculation of the extent of bioavailability (EBA). The various factors that can influence the bioavailability of a drug can be broadly classified as dosage-related or patient-related (interindividual differences such as genetic factors, type of disease, the time and route of administration, the degree of gastric filling, the kind of food or other drugs taken concurrently). Determination of EBA has a very important practical meaning for pharmaceutical companies and medical physicians, enabling appropriate selection of type of preparation, dosage form, route of administration, and dosage of the drug. In cases of ineffective pharmacotherapy, it helps in making a decision about changing the established method of treatment [30].

Often, the determination of even the total concentration of parent drug in biological fluids causes difficulties for the researcher as a result of its low concentration in the tested biological fluids. This, in turn, is usually the result of administrating small-milligram doses of drug. The problem of determination is also complicated by the reduced bioavailability of the active substance in a drug, additionally potentiated by the first pass effect. Very fast elimination of the studied analyte from the body together with slow absorption causes low levels in the body.

Thus, analyte isolation and further concentration are needed to increase the sensitivity of the analytical method, which in turn results in obtaining a correct pharmacokinetic model for a drug or metabolite [31].

10.7.5 Drug Interaction with Other Compounds

Knowledge about the interactions of a drug with other compounds is of a great importance in practice, because it allows the use of favorable interactions and, at the same time, contributes to a reduction in use of combinations that carry a risk of side effects, often very dangerous. In recent years, more and more attention has been paid to the influence of dietary components on the therapeutic effect of drugs used in the treatment of many diseases. The interaction of two compounds occurs when the effect of one drug is changed (i.e., intensifies or weakens) as a result of application of the second.

From a clinical point of view, adverse drug interactions (therapeutically undesirable effects) are particularly important. These interactions reduce or enhance the effects of a drug, causing emergence of toxic symptoms or pharmacological action qualitatively different from that expected. Undesirable interactions require specific control of therapy or even modification of doses, and are a result of a variety of mechanisms and the impact of the patient's individual characteristics on drug metabolism. Sometimes completely unfamiliar or unusual mechanisms underlie two types of reactions, positive or adverse. Most often, however, these reactions are nothing other than repeated, sometimes very well-known, pharmacokinetic processes (pharmacokinetic interactions), most of which are a result of inhibition or induction of metabolic enzymes.

The increasing number of available preparations and dietary supplements, together with intensive advertising campaigns, have generated increased consumption. In addition, taking multiple medications concurrently, without precise knowledge of the mechanisms of their actions, contributes to the intensity of the polypharmacy phenomenon. Risk of polypharmacy is a result of the increased probability of occurrence (or intensity) of adverse drug reactions resulting from interactions between them. Concomitant use of herbal and synthetic medicines increases the risk of dangerous interactions through three mechanisms:

- Impact on the absorption of drugs from the gastrointestinal tract (pharmacokinetic phase)
- Impact on the metabolism of drugs by cytochrome P-450 enzymes (pharmacokinetic phase)
- Additive and hyperadditive synergy (pharmacodynamic phase)

Assessing the impact of dietary ingredients and dietary supplements on the bioavailability of synthetic drugs is still underestimated in the daily work of pharmacists and physicians. Approximately 75 % of drugs are taken orally and almost every component of the diet might affect the drug at one or all stages of its

transition through the body. In the case of concomitant use of several drugs, the number of interactions increases proportionally with the number of drugs used, and if more than five drugs are taken the interactions become disproportionate [32].

Such dependences are confirmed by the statement that the use of population parameters in patients to predict the concentration of a drug can be unreliable. The individual response of a patient to drug use, resulting from genetic polymorphism, determines differences in the final effect of the drug in response to applied supplementation or diet. In this case, monitoring of drug concentrations during therapy, and tracking of their metabolism, seems to be the most reasonable strategy. However, there is a risk that some nutrients or supplements can alter the effect of drugs, depending on different pharmacokinetic processes. This carries important clinical consequences such as lack or reduction in therapeutic effect, or the occurrence of dangerous interactions. Various clinical symptoms occurring during pharmacotherapy could result from taking drugs with food, meaning that the therapeutic effect of a prescribed drug can be unpredictable. The interaction of drugs with other substances taken by a patient, affecting the final result of drug use, is one of the most important problems of modern pharmacotherapy.

Accordingly, more attention is being paid to the impact on therapeutic effect of dietary components and other drugs concurrently prescribed by a doctor. Monitoring of the therapeutic drug is a method of ensuring optimal drug therapy that takes into account differential pharmacokinetics and bioavailability.

In the absence of sufficient knowledge about the risks resulting from drug interactions and dietary components and supplements, it is necessary to undertake studies to define the potential consequences of this type of interaction. Simultaneous determination of drugs and dietary components facilitates the decision-making process regarding individualization of therapy. In the case of drug metabolism interferences, such determinations enable the exclusion or introduction of additional preparations into therapy [33].

The impact of plant products on the metabolism of synthetic drugs results from the inhibition or activation of cytochrome P-450 (CYP) enzymes. Evaluation of the potential activation of CYP by administration of natural plant products or dietary supplements is important for prediction of interactions between their components and drugs. Therefore, attention is directed to research on the impact of products available on the food market known as “natural non-nutritive substances” on drug absorption. Non-nutritive dietary components are mainly secondary plant metabolites, which include, among others, phenolic compounds such as phenolic acids and flavonoids. The health effects of non-nutritive substances are not yet known. So far, there is no answer on the extent to which they are absorbed and metabolized by the body, and there is no information on the permitted daily intake for these compounds. This information is particularly important because certain non-nutritive natural substances are simultaneously considered to be anti-nutritional factors, mainly because they inhibit digestion and reduce the bioavailability of nutrients or drugs. It is also possible that they form undesirable interactions with drugs. The positive health effects of non-nutritive natural substances are not only attributed to their antioxidant properties. These substances are involved in various metabolic

processes and strengthen the body's immune system, which interacts with all other physiological systems, such as respiratory, digestive, nervous, urogenital, and musculoskeletal [34].

The metabolism of drugs involves, among others, CYP2C9, CYP2C8, CYP3A4, and CYP1A2, which might also be involved in the biotransformation of other endogenous and exogenous compounds. The most important role is played by CYP2C9. Its activity can be inhibited or stimulated by many substances. Inhibition of the activity of CYP2X9 can slow metabolism of the drug, thereby increasing its concentration in the body, which can lead to undesirable side effects. An increase in metabolism can result in faster elimination of drug, making achievement of therapeutic concentration impossible. Of the currently used therapeutic substances, over 100 have been identified as substrates for CYP2C9. They represent 10–20 % of all prescribed drugs [35].

In connection with the above, the assessment of potential inhibition of CYP caused by administration of natural plant products and dietary supplements is important for predicting interactions between dietary components and drugs belonging to different treatment groups.

In the face of these needs, there is growing emphasis on the development of new analytical methods enabling fast and efficient analysis of low molecular weight mixtures of organic substances. An important goal is to develop new and effective methods for determination of a large number of chemical compounds, and methods for their detection. At present, methods based on MS play an important role because of their very high sensitivity and selectivity. The use of analytical methods for simultaneous determination of drugs and dietary ingredients creates a real opportunity to eliminate or minimize the interactions between the determined biologically active compounds, and could also provide new information necessary for deciding on treatment procedures. Correlation of the concentration levels of drugs and their metabolites with the concentration of dietary components can help in making a decision about a change of diet and the need to control its supplementation. On the one hand, this type of research provides tools for controlling the progress of therapy for many diseases and, on the other hand, provides a scientific basis for nutritional recommendations aimed at eliminating the possible interactions between drugs and compounds supplied from the diet.

10.7.6 Choice of Analytical Method

According to current clinical practice, only a small part of the information obtained from examination of tissues and biological fluids is used. For disorders of metabolic pathways, reflected in the qualitative and quantitative composition of metabolites of endogenous compounds, analysis of the results of these examinations can be the basis for verification of hypotheses on the causes of such diseases.

Defining the problem is a crucial issue in the development of an appropriate analytical method and determines the choice of sample type and assay methods.

Fundamental issues relate primarily to achieving determinations of the required precision, accuracy, specificity, and detection limit. Due to development of methods for analyzing samples taken from the patient, the limitations considered at the stage of defining the problem are the number and size of samples tested, and the time needed to process and obtain analytical results.

An important factor in selection of analytical method is the type of sample to be analyzed. The main limitation is the amount of the collected test sample. This becomes a very important issue when monitoring the distribution of drugs in infants and children. Additionally, during the selection of test sample, it is necessary to consider whether or not analysis of the material provides the desired information on drug distribution, and whether or not a suitable method of sample storage is applied from the time of collection until analysis. Another important issue is the choice of when to take a sample. This issue becomes truly meaningful with respect to outpatients. The balance between the concentration of substances in body fluids and tissues is achieved after a few hours. Some drugs require administration in the evening and such cases require patient hospitalization and, consequently, affect the economic issues of hospitals. To sum up, it is necessary to select an appropriate sample size for analysis, storage of which from the time of collection until analysis must not affect the concentration of the analyte. In addition, the sample should be able to provide the desired information at a time of collection convenient for the laboratory [36].

The type of analyzed sample significantly reduces the choice of appropriate analytical method as a result of the presence of a complex matrix. When choosing a method of analysis, consideration should also be given to the range of concentrations possible for determination by a particular method and the drug content in real samples at the usually adopted dosage regimens. There should be no change in the analyzed substance as a result of metabolism of the taken drugs and the multitude of metabolites produced in the body during preparation for analysis. In addition, the method selected should allow determination of both the dosage form of the drug and its metabolites. Application of the chosen analytical method requires development of appropriate methods of sample preparation. The selected processing method should eliminate the influence of the matrix, achieve analyte concentration, and not cause any changes in the structure of the analyzed substances, whether administered drug or its metabolites.

After selecting the appropriate material for the research and development of processing methods, the samples are subjected to analysis. The last stage of research is interpretation of results and drawing conclusions about the patient's condition.

In the literature, pharmacokinetic studies of drugs and their metabolites are described using appropriately sensitive and selective analytical methods. The vast majority of the methods used involve LC techniques. The combination of chromatography with MS is increasingly used in the determination of many compounds, including drugs belonging to different treatment groups. In the numerous described procedures for determination of pharmaceuticals in biological fluids, spectrophotometric (UV-vis), fluorescent, and MS or MS/MS detectors have been used.

For separation are used stationary phases with nonpolar groups (C18), but also with others, including hydrophilic interaction liquid chromatography (HILIC). Mixtures of acetonitrile, methanol, and water in different ratios are used as eluents, often with the addition of acid or amine to change the pH. This has an impact on the protonation or deprotonation of the examined analytes. Chromatographic analyses of biological fluid solutions to detect drugs and their metabolites are also carried out using chiral stationary phases, and mixtures of acetonitrile or methanol with various buffers as mobile phases. Gas chromatography is also often used in combination with various types of detectors. An alternative to conventional techniques of chromatographic separation is to use capillary electromigration chromatographic techniques, of which capillary zone electrophoresis (CZE) is particularly effective.

Because of the complex nature of biological material, it is necessary to separate the analyte from both endogenous matrix-derived substances and other compounds that could affect the assay values. Moreover, the sample preparation procedure should include, in addition to isolation of analytes from biological material, their derivatization to increase the sensitivity of the method. Chemical conversion of analytes into appropriate derivatives with a higher capacity than the parent compound for fluorescence or UV absorption is particularly important when determining low levels of drug in biological fluids. Because endogenous compounds and other drugs with a similar structure to the analyte can also undergo derivatization, it is necessary to apply selective isolation of the drug and its metabolites in biological matrices by derivatization, and also to separate created analyte derivatives from other derivatization products that could interfere with the determined compounds [37].

New methodological and instrumental solutions are also necessary for obtaining reliable information on the pharmacokinetics of drugs and determining the level of their bioavailability in response to a diet rich in polyphenolic compounds. Use of these newer analytical methods facilitates acquisition of additional, relevant information, which is important in making treatment decisions regarding the use of new drugs. This, in consequence, increases the effectiveness and safety of pharmacotherapy for many diseases. Only knowledge of pathopharmacokinetics (science dealing with the impact of pathological conditions on the fate of a drug in the body) can effectively predict the concentration of a drug in the blood. Furthermore, interactions between drugs and other compounds can be measured by observing the condition of the patient and any additional adverse effects. The results of such interactions are pharmacokinetic and pharmacodynamic parameters measureable in absolute terms; precise mathematical characterization of drug interactions enables modeling of the pharmacokinetic/pharmacodynamic type. Understanding the pharmacokinetic properties of analyzed drugs gives a real chance to eliminate or minimize interactions with other active substances. Taking into account the results of pharmacokinetic studies of selected compounds in the complex process of patient care can be extremely important for clinical purposes and provide a basis for the individualization of therapy [38].

10.7.7 Procedures and Analytical Techniques

Determination of drugs and their metabolites is a difficult analytical problem because of the strong similarity of the chemical structures of these two groups of compounds. A similar structure results in similar physicochemical properties and, hence, similar interactions of the analyzed compounds with the phases of chromatography systems. The similarity of properties results in similar parameters for analytical signals, for example, similar wavelength of light in spectrophotometric detection or similar values for oxidation or reduction potentials in voltammetric detection. If the metabolites are present in the form of sulfates or glucuronides, the separation is easier because it is possible to force or prevent dissociation processes simply by changing the pH of the mobile phase in chromatographic methods. This results in diversity of the interactions of the compounds (in the form of neutral molecules and ions) with the stationary and mobile phases in the chromatographic system. Similarly, protonation and deprotonation processes (e.g., of amino groups in drugs and metabolites) simplify chromatographic separation.

The available literature describes a number of methods for determination of specific drugs in biological fluids without the simultaneous determination of metabolites. There is also extensive literature available regarding drug analysis, so this review presents only some methods for simultaneous determination of drugs and their metabolites. Literature data described in this chapter refers to simultaneous determination of drugs and their metabolites. Table 10.1 describes methods for determination of selected anti-inflammatory drugs and analgesics applied in cardiovascular diseases, and their metabolites.

An important problem in this type of analysis is the presence of a matrix, the components of which hamper analysis by falsifying the results or generally making determination impossible. Therefore, in addition to developing appropriate methods of analysis, it is necessary to remove interferents and also to isolate and enrich analytes. For this purpose, various types of extractions are applied, usually LLE and SPE, but also others such as microextraction by packed sorbant (MEPS) and ultrasound-assisted emulsification microextraction (USAEME).

In the case of polytherapy, it is necessary to know the possibilities of drug–drug interactions, their mechanisms of action, and possible influences on the pharmacokinetics of drugs. Polypharmacy phenomena occur when, in addition to drugs prescribed by a physician, patients also take other medications as a result of the high availability of non-prescription pharmaceuticals. Drug interactions can result in increased toxicity and severe complications for therapy.

These above-mentioned phenomena require the application of analytical methods that allow simultaneous monitoring of the presence and concentrations of drugs from different therapeutic groups in body fluids. There are a few such methods described in the available literature and examples are presented in Tables 10.2 and 10.3.

Table 10.1 Chromatographic methods for the determination of selected drugs and metabolites

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Non-steroidal anti-inflammatory drugs					
Paracetamol, metabolites/blood	HPLC UV	Column: Hypersil C_{18} (75 × 4.6 mm, 3 μm) Mobile phase: Solvent A: 20 mM ammonium formate buffer (pH 3.5) Solvent B: MeOH (gradient elution) Detection: $\lambda = 254$ nm	Extraction LLE/20 mM ammonium formate buffer (pH 3.5)	LOD: 0.03 μg/mL (paracetamol); 0.1 μg/mL (metabolites)	[39]
Paracetamol, metabolites/urine, plasma	HPLC UV	Column: EPS C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: 0.1 M KH_2PO_4: isopropanol:THF (100:1.5:0.1 pH 3.7), adjusted with phosphoric acid (isocratic elution) Detection: $\lambda = 254$ nm	Plasma: protein precipitation (30 % perchloric acid) Urine: 20 times dilution, centrifugation	LOQ: 0.06 μg/mL (paracetamol); 0.13 μg/mL (metabolites)	[40]
Paracetamol, metabolites/urine	HPLC NMR/MS	Column: YMC-Pack J'Sphere H80 (250 × 2 mm, 4 μm) Mobile phase: Solvent A: 0.1 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 254$ nm, MS, ionization ESI, NMR	On-line SPE	–	[41]
Paracetamol, metabolites/urine	UPLC MS	Column: ChromSpeed (50 × 4.6 mm, monolithic) Mobile phase: Solvent A: 0.1 % HCOOH in water Solvent B: 0.1 % HCOOH in ACN (gradient elution) Detection: MS, ionization ESI	Five times dilution	–	[42]

Aspirin, salicylic acid, gentisic acid/plasma	HPLC UV	Column: YMC Hydrosphere C_{18} (150 × 4.6 mm, 5 μm) Mobile phase: Solvent A: water:ACN (1000:10 v/v) with 0.2 % TFA Solvent B: water:ACN (100:900 v/v) with 0.2 % TFA (gradient elution) Detection: $\lambda = 235$ nm	On-line SPE/MCX-SAX column	LOQ: 60 ng/mL	[43]
Acetylsalicylic acid, ketoprofen, diclofenac, naproxen and ibuprofen/urine	UHPLC UV	Column: Poroshell 120 EC-C18 (100 × 3.0 mm; 2.7 μm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 221$, 230, 239, 255, 277 nm	Extraction MEPS/sorbent C18/elution: ACN	LOD: 1.07–16.2 ng/mL LOQ: 3.21–48.7 ng/mL	[44]
1-Hydroxy-ibuprofen, 2-Hydroxy-ibuprofen, 3-hydroxyibuprofen, carboxyibuprofen/urine	UHPLC MS/MS	Column: Zorbax Rapid Resolution High Definition (RRHD) SB-C18 (50 × 2.1 mm, 1.8 μm) Mobile phase: Solvent A: 0.1 % HCOOH in water Solvent B: ACN (gradient elution) Detection: MS/MS, ionization ESI	Extraction USAEME/1-octanol	LOQ: 0.5 pg/mL	[45]
Flurbiprofen, ketoprofen, etodolac enantiomers/plasma	HPLC UV	Column: Agilent Zorbax C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: ACN:KH_2PO_4 (pH 4.5) (60:40 v/v) (isocratic elution) Detection: $\lambda = 250$ nm	Extraction LLE/dichloromethane, derivatization	LOD: 0.15 μg/mL LOQ: 0.5 μg/mL	[46]
Ketoprofen enantiomers/plasma	HPLC UV	Column: Chiral-HSA (100 × 4 mm, 5 μm) Mobile phase: 0.01 M phosphate buffer:2-propanol (94:6 v/v) with 5 mM octanoic acid, pH 5.5 (isocratic elution) Detection: $\lambda = 260$ nm	On-line SPE/column LiChrospher C_{18} ADS	LOQ: 16 ng/mL	[47]

(continued)

Table 10.1 (continued)

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Diuretics					
Spironolactone, canrenone/plasma	HPLC DAD	Column: Waters Symmetry C18 (150 × 4 mm, 5 µm) Mobile phase: MeOH:water (60:40 v/v) (isocratic elution) Detection: $\lambda = 238$ nm (spironolactone); $\lambda = 280$ nm (canrenone)	Extraction SPE/column Oasis HLB/elution MeOH	LOQ: 28 ng/mL (spironolactone); 25 ng/mL (canrenone)	[48]
Spironolactone, canrenone, furosemid/urine	HPLC ESI MS/MS	Column: Waters Sunfire C18 (50 × 2.1 mm, 3.5 µm) Mobile phase: MeOH:water (both containing 1 mM NH_4Ac/0.001 % CH_3COOH) (gradient elution) Detection: MS/MS, ionization ESI	Centrifugation	LOD: 62.5 ng/mL (furosemid); 25 ng/mL (spironolactone, canrenone)	[49]
Spironolactone, canrenone, furosemid/urine	HPLC MS/MS	Column: Altech Prevail C18 (50 × 2.1 mm, 3.0 µm) Mobile phase: Solvent A: 0.2 % HCOOH in water, Solvent B: water, Solvent C: ACN (gradient elution) Detection: MS/MS, ionization APCI	Extraction SPE/column ABS ELUT Nexus/elution: MeOH	LOD: 3 ng/mL (canrenone, spironolactone); 12 ng/mL (furosemid)	[50]
Canrenone, furosemid/urine	HPLC MS	Column: Zorbax XDB C8 (75 × 4.6 mm, 3.5 µm) Mobile phase: Solvent A: 0.2 mM NH_4Ac in water:ACN (95:5 v/v) Solvent B: 0.2 mM NH_4Ac in water: ACN (5:95 v/v) (gradient elution) Detection: MS, ionization APCI	Extraction SPE/column XAD/elution: MeOH	–	[51]

β-Blockers					
Metoprolol, α-hydroxymetoprolol, *O*-desmethylometoprolol/plasma	HPLC FL	Column: μBondpak Phenyl (300 × 3.9 mm, 10 μm) Mobile phase: phosphate buffer (pH 3.5):ACN (85:15 v/v) (isocratic elution) Detection: $\lambda_{EX/EM} = 277/305$ nm	Extraction LLE/diethyl ether: chloroform (4:1 v/v)	LOQ: 25 ng/mL	[52]
Milrinone, sotalol, metoprolol, propranolol and carvedilol, and their metabolites: 5′-hydroxylphenyl-carvedilol, *O*-desmethyl-carvedilol, 4-hydroxy-propranolol, α-hydroxy-metoprolol, *O*-desmethyl-metoprolol/urine	UHPLC UV	Column: Hypersil GOLD™ (50 × 2.1 mm, 1.9 μm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 227$, 240, 254, 280, 324 nm	Extraction SPE/column Oasis HLB/elution: MeOH:acetone: HCOOH (4.5:4.5:1 v/v/v)	LOD: 12.8–37.9 ng/mL LOQ: 38.4–113.8 ng/mL	[53]
Milrinone, sotalol, metoprolol, propranolol, carvedilol, 5′-hydroxylphenyl-carvedilol, *O*-desmethyl-carvedilol, 4-hydroxy-propranolol, α-hydroxy-metoprolol, *O*-desmethyl-metoprolol/urine	UHPLC MS/MS	Column: Hypersil GOLD™ (50 × 2.1 mm, 1.9 μm) Mobile phase: Solvent A: 0.1 % HCOOH in water Solvent B: ACN (gradient elution) Detection: MS/MS, ionization ESI	Protein precipitation	LOQ: 0.05–40 ng/mL	[54]

(continued)

Table 10.1 (continued)

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Milrinone, sotalol, metoprolol, propranolol, carvedilol/urine	UHPLC UV	Column: Hypersil GOLD™ (50 × 2.1 mm, 1.9 μm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 227, 254, 280, 324$ nm	Extraction SPE/column Oasis HLB/elution: MeOH:acetone: HCOOH (45:45:10 v/v/v)	LOD: 10.3–23.7 ng/mL LOQ: 30.9–71.0 ng/mL	[55]
Propranolol, 4-hydroxy-propranolol/plasma	HPLC MS/MS	Column: LiChrospher 60 RP Select B (125 × 4 mm, 5 μm) Mobile phase: 1 mM ammonium formate in water (pH 3.1):ACN (20:80 v/v) (isocratic elution) Detection: MS/MS, ionization ESI	Extraction SPE/cloumn Oasis HLB/elution: MeOH:acetone: HCOOH (45:45:10 v/v/v)	LOQ: 0.2 ng/mL	[56]
Metoprolol, α-hydroxymetoprolol enantiomers/plasma	HPLC FL	Column: Chirobiotic T (250 × 4.6 mm, 5 μm) Mobile phase: ACN:MeOH:CH_2Cl_2: GAA:TEA (50:30:14:2:2 v/v/v/v/v) (isocratic elution) Detection: $\lambda_{EX/EM} = 225/310$ nm	Extraction SPE/column C2/0.1 M HCl:ACN (50:50 v/v)	LOQ: 0.5 ng/mL (metoprolol); 1.0 ng/mL (α-hydroxy-metoprolol)	[57]
Metoprolol enaantiomers/plasma	HPLC FL	Column A: Chiralpak AD (250 × 4.6 mm, 5 μm) Column B: Chirarcel OD-H (150 × 4.6 mm, 5 μm) Mobile phase: Solvent A: hexane: isopropanol:DEA (95:5:0.1 v/v/v) Solvent B: *n*-hexane: EtOH:2-propanol:DEA (88:10.2:1.8:0.2 v/v/v/v) (isocratic elution) Detection: $\lambda_{EX/EM} = 229/298$ nm	Extraction LLE/dichloromethane: diisopropyl ether (1:1 v/v) Extraction SPE/column C18/elution: MeOH	LOQ: 5 ng/mL	[58]

Metoprolol enantiomers/urine	HPLC FL	Column: A: Phenomenex Silica (250 × 4.6 mm, 5 μm) Column B: Chiracel OD (250 × 4.6 mm, 5 μm) Mobile phase: *n*-hexane:EtOH:2-propanol: DEA (90:5:5:0.5 v/v/v/v) (isocratic elution) Detection: $\lambda_{EX/EM} = 276/309$ nm	Extraction LLE/dichloromethane	LOQ: 0.1 μg/mL	[59]
Metoprolol enantiomers/urine	HPLC MS	Column: Chirobiotic T (250 × 4.6 mm, 5 μm) Mobile phase: MeOH:CH_3COOH:NH_3 (100:0.15:0.15 v/v/v) (isocratic elution) Detection: MS, ionization ESI	Extraction LLE/ethyl acetate	LOQ: 0.5 ng/mL	[60]
Carvedilol, 5′-hydroxyphenyl-carvedilol enantiomers/urine	HPLC FL HPLC MS/MS	Column: CHIRALCEL® OD-RH (150 × 4.6 mm; 5 μm) Mobile phase: Solvent A: 0.05 % TFA, 0.05 % DEA in water Solvent B: ACN Detection: $\lambda_{EX/EM} = 254/356$ nm MS/MS, ionization ESI	Extraction SPE/column Oasis HLB/elution: MeOH:acetone: HCOOH (4.5:4.5:1 v/v/v)	LOD: 4.73–8.07 ng/mL LOQ: 14.2–24.2 ng/mL	[61]
Propranolol enantiomers/plasma	HPLC MS/MS	Column: Chirobiotic V (250 × 4.6 mm, 5 μm) Mobile phase: MeOH:CH_3COOH: TEA (gradient elution) Detection: MS/MS, ionization APCI	Extraction SPE/column Oasis MCX/2.25 % NH_4OH in MeOH	LOD: 0.03 ng/mL	[62]
Sotalol enantiomers/plasma	HPLC FL	Column: Chiral-CBH (150 × 4.0 mm, 5 μm) Mobile phase: 15 % 2-propanol in 10 mM phosphate buffer (pH 7.0) including 0.05 mM EDTA (isocratic elution) Detection: $\lambda_{EX/EM} = 250/312$ nm	Extraction on-line SPE/column LiChroCart C18	LOD: 18 ng/mL LOQ: 37 ng/mL	[63]

(continued)

Table 10.1 (continued)

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Other drugs					
Dexamethasone, 6-β-hydroxy-dexamethasone/urine	HPLC DAD	Column: Nova-Pak C_{18} (400 × 3.9 mm, 4 μm) Mobile phase: Solvent A: 0.06 % trifluoroacetic acid in ammonium acetate buffer (0.01 M, pH 4.8):ACN (90:10 v/v) Solvent B: 00.06 % trifluoroacetic acid in ammonium acetate buffer:acetonitrile (30:70 v/v) (gradient elution) Detection: $\lambda = 245$ nm	Extraction SPE/column Oasis HLB/ethyl acetate + diethyl ether	LOQ: 10 ng/mL (dexamethasone) 25 ng/mL (6β-hydroxy-dexamethasone)	[64]
Nifedipine, dehydronifedipine/plasma	HPLC MS/MS	Column: Hypersil BDS C18 (50 × 2.1 mm, 3 μm) Mobile phase: MeOH:1 % TFA in water (80:20 v/v) (isocratic elution) Detection: MS, ionization ESI	Extraction LLE/diethyl ether: *n*-hexane	LOQ: 0.5 ng/mL	[65]
L-arginine, metabolites/urine	HPLC FL	Column: Purospher® STAR RP-18e (250 × 4 mm, 5 μm) Mobile phase: Solvent A: di-sodium hydrogen phosphate/potassium hydrogen phosphate, pH 6.88 Solvent B: ACN Solvent C: MeOH (gradient elution) Detection: $\lambda_{EX/EM} = 338/455$ nm	Extraction SPE/column Oasis MCX/elution: 25 % NH_3:water: MeOH (10:40:50 v/v/v)	LOD: 0.05–198.5 pmol/20 μL injection LOQ: 0.17–655.1 pmol/20 μL injection	[66]

Table 10.2 Chromatographic methods in the determination of selected drugs from different therapeutic groups in biological material

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Acenocoumarol, phenprocoumon enantiomers/plasma	HPLC UV	Column: S,S-Whelk-01 (250 × 4 mm, 5 μm) Mobile phase: Solvent A: hexane:EtOH with CH_3COOH Solvent B: hexane:EtOH (gradient elution) Detection: $\lambda = 310$ nm	Extraction LLE/toluene	LOD: 5 ng/mL	[67]
Acenocoumarol, phenprocoumon, warfarin enantiomers/plasma	HPLC MS MS	Column: Chira-Grom-2 (250 × 1 mm, 8 μm) Mobile phase: ACN:MeOH: CH_3COOH (gradient elution) Detection: MS/MS, ionization ESI	Extraction on-line SPE/column Poros R2/20 (2 × 30 mm)	LOD: 0.5 ng/mL LOQ: 2 ng/mL	[68]
Aliskiren, prasugrel, rivaroxaban/urine	UHPLC MS/MS	Column: Zorbax Rapid Resolution High Definition SB-C18 column (50 × 2.1 mm, 1.8 μm) Mobile phase: 0.1 % HCOOH in water: ACN (70:30 v/v) (isocratic elution) Detection: MS/MS, ionization ESI	Extraction MEPS/ sorbent C8/elution: MeOH	LOQ: 0.5–5 pg/mL	[69]
Aliskiren, enalapril, enalaprilat/urine	UHPLC MS/MS	Column: Poroshell 120 EC-C18 (100 × 2.1 mm; 2.7 μm) Mobile phase: Solvent A: 0.1 % HCOOH in water Solvent B: ACN (gradient elution) Detection: MS/MS, ionization ESI	Extraction MEPS/ sorbent C8/elution: MeOH	LOQ: 0.01 ng/mL	[70]
Sotalol, metoprolol, propranolol, carvedilol, nifedipine, captopril, cilazapril, milrinone, ticlopidine, acenocoumarol, furosemide, acetylsalicylic acid, salicylic acid, ibuprofen, naproxen, ketoprofen, diclofenac, paracetamol, dipyrone, mildronate, sildenafil, dexamethasone, carbamazepine, terbinafine/urine	UHPLC MS/MS	Column: Zorbax Rapid Resolution High Definition SB-C18 (50 × 2.1 mm, 1.8 μm) Mobile phase: Solvent A: 0.1 % HCOOH in water Solvent B: MeOH (gradient elution) Detection: MS/MS, ionization ESI	Protein precipitation	LOQ: 0.05–0.60 ng/mL	[71]

(continued)

Table 10.2 (continued)

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Enalapril, paracetamol, sotalol, dipyrone, vancomycin, captopril, fluconazole, cefazolin, metoprolol, aspirin, ticlopidine, prednisolone, propranolol, digoxin, sildenafil, furosemide, dexamethasone, carvedilol, ketoprofen, nifedipine, terbinafine, acenocoumarol, spironolactone/urine	HPLC DAD	Column: LiChroCART® Purospher® STAR, RP-18e (250 × 4 mm, 5 µm) Mobile phase: MeOH:ACN:0.05 % TFA in water (gradient elution) Detection: DAD $\lambda = 200–450$ nm	Adjusted to pH 7.0, protein precipitation	LOD: 0.01–1.44 µg/mL LOQ: 0.04–4.35 µg/mL	[72]
Imipenem, paracetamol, dipyrone, vancomycin, amikacin, fluconazole, cefazolin, prednisolone, dexamethasone, furosemide, ketoprofen/urine	HPLC DAD	Column: LiChroCART Purospher STAR, RP-18e (125 × 3 mm, 5 µm) Mobile phase: MeOH:ACN:0.05 % TFA in water (gradient elution) Detection: DAD $\lambda = 200–450$ nm	Adjusted to the pH 8.0, protein precipitation	LOD: 0.01–1.15 µg/mL LOQ: 0.03–3.75 µg/mL	[73]
Amikacyna, cefazolin, deksametazon, desmetylosildenafil, dipyron, flukonazol, furosemid, imipenem, ketoprofen, paracetamol, prednizolon, sildenafil wankomycyna/urine	HPLC DAD	Column: LiChroCART® Purospher® STAR, RP-18e, (125 × 3 mm, 5 µm) Mobile phase: ACN: buffer (acetic acid, sodium acetate, pH 4.66):0.05 % TFA in water (gradient elution) Detection: DAD $\lambda = 200–450$ nm	Extraction LLE/acetate: dichloromethane: chloroform (45:35:20 v/v/v)	LOD: 0.01–1.16 µg/mL LOQ: 0.02–3.45 µg/mL	[74]
Aliskiren, prasugrel, rivaroxaban, prednisolone, propranolol, ketoprofen, nifedipine, naproxen, terbinafine, ibuprofen, diclofenac, sildenafil, acenocoumarol/urine	UHPLC UV	Column: Poroshell 120 EC-C_{18} (100 × 3.0 mm; 2.7 µm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 221, 228, 230, 240, 250, 275, 280$ nm	Extraction SPE/column C_6H_5/elution: MeOH	LOD: 0.003–0.217 µg/mL LOQ: 0.01–0.650 µg/mL	[75]

Milrinone, enalapril, carvedilol, spironolactone, acenocumarol, ticlopidine, cilazapril, 2-oxoticlopidine, cilazaprilat, canrenone, 5′-hydroxycarvedilol, *O*-desmethylcarvedilol, enalaprilat/urine	UHPLC UV	Column: Poroshell 120 EC-C_{18} (100 × 3.0 mm; 2.7 μm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 227$, 235, 255, 269, 285, 324 nm	Extraction MEPS/ sorbent C18/elution: MeOH:ACN (50:50 v/v)	LOQ: 0.016–0.045 μg/mL	[76]
Acetylsalicylic acid, caffeine, paracetamol/ plasma, urine	HPLC UV	Column: μBondapak C_{18} (300 × 3.9 mm, 10 μm) Mobile phase: Solvent A: ACN Solvent B: water (gradient elution) Detection: $\lambda = 280$ nm	Extraction SPE/column C_{18}/elution: MeOH	LOD: 0.1–0.2 μg/ mL LOQ: 0.15–0.2 μg/ mL	[77]
Deksametazon, prednisone, prednisolone, dexamethasone, cortisol/serum	HPLC MS MS	Column: Symmetry C_{18} (30 × 2.1 mm, 3.5 μm) Mobile phase: MeOH:5 mM acetate buffer, pH 3.25 (gradient elution) Detection: MS/MS, ionization ESI	Extraction SPE/column Oasis HLB/elution: MeOH	LOD: 0.20–0.58 ng/mL LOQ: 5.4–10.7 ng/ mL	[78]
Sotalol, metoprolol, propranolol, carvedilol, salicylic acid, dexamethasone, prednisolone, ketoprofen/urine	UHPLC UV	Column: Chromolith Fast Gradient Monolithic RP-18e (50 × 2 mm) Mobile phase: Solvent A: 0.05 % TFA in water Solvent B: ACN (gradient elution) Detection: $\lambda = 227$, 240, 254 nm	Extraction SPE/column SDB/elution: MeOH	LOD: 11.8–42.2 ng/mL LOQ: 35.6–126.7 ng/mL	[79]
19 diuretics/urine	HPLC UV	Column: Hypersil (150 × 3.0 mm, 5 μm) Mobile phase: sodium dodecyl sulfate (SDS):PrOH:BuOH:PenOH:ACN or SDS:THF:phosphoric acid (gradient elution) Detection: $\lambda = 245$, 220 nm	Filtration (0.45 μm)	LOD: 1–39 ng/mL	[80]

(continued)

Table 10.2 (continued)

Analyte/sample	Technique	Chromatographic conditions	Sample preparation	LOD/LOQ	Reference
Cefepime, vancomycin, imipenem/plasma	HPLC UV	Column: Supelcosil LC-18 (250 × 4.6 mm, 5 µm) Mobile phase: 0.075 M acetate buffer (pH 5.0):ACN (92:8 v/v) Detection: $\lambda = 230$ nm	Extraction LLE/3-(*N*-morpholino) propanesulfonic acid	LOD: 0.17–0.38 µg/mL LOQ: 0.4–0.76 µg/mL	[81]
Prednisolone, cortisol/urine	HPLC UV	Column: Thermo Hypersil (250 × 4.6 mm, 5 µm) Mobile phase: dichloromethane:water:MeOH:THF (66.5:30:2.5:1 v/v/v/v) Detection: $\lambda = 240$ nm	Extraction SPE/column Oasis HLB/elution: MeOH	LOD: 4.8–7 ng/mL LOQ: 9.9–11.6 ng/mL	[82]

Table 10.3 Voltammetric methods in the determination of selected drugs

Analyte/sample	Technique	Voltammetric conditions	Sample preparation	LOD/LOQ	Reference
Cephalexin, cefazolin/human synthetic serum	DPV	Working electrode: GCE Reference electrode: Ag/Ag/Cl Supporting electrolyte: 0.1 M phosphate buffer solution (pH 3.0)	Dissolving in the supporting electrolyte	LOD: 1×10^{-6} M LOQ: n/a	[83]
Paracetamol, its glucuronide, sulfate metabolites/model solutions	DPV	Working electrode: GCE Reference electrode: Ag/Ag/Cl Supporting electrolyte: Britton-Robinson buffer (pH 3.29)	Dissolving in the supporting electrolyte	LOD: 3.27–5.09 μM LOQ: 1.09–1.70 μM	[84]
Propranolol, 4′-hydroxypropranolol, 4′-hydroxypropranolol sulfate/urine	DPV	Working electrode: GCE, MWCNT-GCE Reference electrode: Ag/Ag/Cl Supporting electrolyte: Britton-Robinson buffer (pH 3)	Extraction SPE/column Oasis HLB/elution: MeOH	LOD: 1.10–1.37 μmol/L LOQ: 3.31–4.11 μmol/L	[85]
Paracetamol, furosemide, dipyrone, cefazolin and dexamethasone/urine	DPV	Working electrode: HMDE and graphite Reference electrode: Ag/Ag/Cl Supporting electrolyte: Britton-Robinson buffer (pH 2.4)	Extraction SPE/column SPE NH_2/elution: MeOH Extraction LLE/ethyl acetate	LOD: 0.20–2.57 μM LOQ: 0.53–6.28 μM	[86]
Enalapril, lisinopril/tablets	DPP	Working electrode: MME Reference electrode: Ag/Ag/Cl Supporting electrolyte: borate buffer (pH = 9 and 10)	Derivatization/2,4-dinitrofluorobenzene	LOD: 0.004–0.6 μg/mL LOQ: 0.14–2.00 μg/mL	[87]
Carvedilol, paracetamol, sildenafil/urine	DPV	Working electrode: GCE Reference electrode: Ag/Ag/Cl Supporting electrolyte: Britton-Robinson buffer (pH 3.29)	Extraction SPE/column Oasis MCX/elution: dichloromethane:2-propanol:ammonium (78:20:2 v/v/v)	LOD: 2.5–5.0 μg/mL LOQ: –	[88]
Ascorbic acid, acetaminophen/tablets	LSV	Working electrode: carbon paste electrode Reference electrode: Ag/AgCl Supporting electrolyte: acetate buffer (pH 4.7)	Dissolving in water	LOD: – LOQ: –	[89]

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Chapter 11
Forensic Analytics

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11.1 Criminalistic Analytics

Janina Zięba-Palus

Analysis of various materials for forensic purposes is known as trace analysis. The problem in this field tends not to be the small quantity (concentration) of the analyte in question, but rather the small quantity of material (forming the criminalistic trace at the scene of the crime or incident) that is available for examination. Its mass is often on the order of milligrams or micrograms. This trace amount of disclosed material (e.g. skin fragments, individual fibres, dust, pieces of glass and plastic, soil particles or droplets of blood) constitutes a valuable source of information about the event and persons taking part in it. Disclosure and appropriate securing of material from the scene of the incident is thus crucial for carrying out examinations correctly in the criminalistic laboratory and for explaining the circumstances of the crime.

11.1.1 Concept of a Criminalistic Trace

The concepts of trace and microtrace were introduced into criminalistics to define material that had been found at the scene of a crime and then subjected to examination. The concept of a criminalistic trace was defined by Jan Sehn, who stated that "traces in the criminalistic sense are changes in objective reality (. . .)

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after events that are under investigation", which "may constitute a basis for recreating and establishing the course of these events in accordance with what actually happened" [1]. As understood by this definition, traces are the consequences of some behaviours and phenomena, and thus exhibit a causal link with these behaviours or phenomena. A trace is thus an object or part of an object left at the scene of an incident, an impression of the sole of a shoe or tyre, a dent or scratch on a surface caused by a tool, a fingerprint, a liquid stain (e.g. blood, oil), a hair, a sliver of glass or paint, or a change in the shape of an object caused by heat or force. Traces are perceived via the senses or with the use of technical devices to aid these senses (magnifying glass, microscope, illuminator, etc.). Traces have a material (physical) nature and are thus possible to detect and study.

The importance of traces lies mainly in their reconstructive potential; on the basis of traces it is possible to recreate the course of a particular incident and determine which persons took part in it, as well as their behaviour at the time of the event. Furthermore, traces can be useful aids in enabling direct apprehension of the perpetrator of an incident. Examination of traces can also indicate whether and how defined persons were linked to an event that is of interest to a judicial body. Traces can also play a significant role in the identification of places, persons and things.

Each criminalistic trace can occur at the scene of an incident in various sizes. Advances in measuring equipment, new analytical chemistry techniques and empirical research in this field, as well as the small number of disclosed macrotraces, have led to microtraces acquiring particular importance. In physicochemical terms, microtraces do not differ from macrotraces. They are particles of matter weighing milligrams or less (e.g. soil particles, dust, microfibres, skin fragments and liquid droplets). They can also be gases that are undetectable by the sense of smell or microscopic marks of mechanical action in the form of scratches, dents, or cracks that are invisible or hardly visible to the naked eye. Mirosław Owoc defined them as follows: "Microtraces are those criminalistic traces, which, due to their small dimensions or other particular properties are, without appropriate observational instruments, imperceptible or poorly perceptible by humans, and can only be examined by applying microanalytical methods" [1]. The size of microtraces means that, as a rule, various microscopic and microanalytical techniques must be used to examine them. Impurities and additives are also considered to be microtraces and must be studied using advanced analytical methods.

11.1.2 Features of Microtraces

Microtraces are characterised not only by their microscopic size, but also by their prevalence, the fact that perpetrators are unable to avoid leaving them (irrespective of perpetrators' efforts) and the difficulty in removing them. Microtraces arise as a result of the interactions of the perpetrator (his/her clothes, tools/instruments used) with the surroundings. Most frequently, microtraces are small particles of an originally larger object that have separated from it, such as microfibres, particles

of glass, paint, metal, plastic, soil or explosives. Where macrotraces occur, microtraces also usually occur, constituting a sort of supplement to them. However, from the point of view of criminalistic practice, cases in which there is a lack of macrotraces because they have been destroyed or removed by the perpetrator or other persons, or an experienced criminal has not left any, microtraces are much more valuable. In principal, it is impossible to avoid leaving microtraces. Microtraces occur at the scene of every event, on every tool that the perpetrator has used, as well as on the victim and on objects belonging to him/her. Dust particles or fibres accumulate easily in recesses and cracks of a substrate; hence, it is difficult to remove them. Microtraces are thus "resistant" to destructive factors (e.g. washing and wiping). The action of adhesive forces additionally helps to keep microtraces on the surface of a substrate. In terms of chemical composition, microtraces are usually mixtures of many compounds.

Because of the (tiny) size of microtraces, they are often unconsciously destroyed; for example, they can be accidentally lost or transferred to another substrate, evaporate at elevated temperatures, or destroyed by the action of fire. Their properties might also change. The time elapsed between leaving a trace and its disclosure and securing for examination, as well as atmospheric conditions, are sometimes the cause of chemical and biological breakdown of the trace, or the substrate on which it occurs, making it difficult to carry out analyses.

11.1.3 Difficulties in the Study of Criminalistic Traces

There are many factors that significantly affect the possibility of carrying out analyses of disclosed traces, the choice of analytical methods and interpretation of the obtained results. Among the basic factors are the goal of the examination and the applied methods of disclosure.

11.1.3.1 Aim of the Examination

The aim of chemical analysis of traces is, above all, their identification. Currently, there are many analytical methods and research techniques enabling identification of a wide variety of materials with great accuracy and precision. Qualitative determination of the chemical composition of studied materials and establishing their type does not usually present difficulties, irrespective of the size of the studied sample. In the case of materials such as paints or fibres, the availability of broad databases for reference purposes allows their producer to be identified. However, information obtained about materials in the course of analyses leads only to group identification and not individual identification. This means that, as a result of conducted analyses, the material can be classified into a group of objects (materials) of the same composition and properties, bearing in mind that the size of such a group varies and depends on the amount of information obtained during analysis.

Analytical chemistry methods can help to elucidate whether materials being compared are the same or whether they differ from each other. However, such methods cannot establish whether a studied sample is part of one and the same material system, and thus whether it is of the same material. Therefore, these methods cannot individually identify secured materials. Individual identification is, of course, possible, but achieving it requires going beyond determination of the chemical composition and physicochemical properties of a trace.

Traces are identified by determining their chemical composition and certain physicochemical properties, and comparison with reference material (database) or comparative material collected from the suspect. Criminalistic laboratories create extensive databases on the physical features and production of glass, paints, papers, fabrics and other materials, as well as collecting thousands of samples for comparison purposes.

11.1.3.2 Material

Microtraces are usually very small samples (e.g. milligram or microgram weight) of material such as pieces of paint, a few drops of oil, individual fibres, a hair or fragments of glass or plastic. Once this material has been secured at the scene of the incident, it cannot be collected again for examination. It is therefore quantitatively limited and must suffice for all ordered examinations, and some of it should also be preserved. Furthermore, samples submitted to a laboratory are usually contaminated and often difficult to separate from the substrate on which they occur. The smaller the dimensions of the studied materials and the more subtle their structure, the greater is the influence of the substrate on the results of analyses. Thus, if it is not possible to separate the studied material from the substrate, then the effect of the substrate on the results of analysis should be taken into account. An example might be an examination of initials, signed to confirm receipt of money. Initials are composed of, at most, several letters that have been executed, for example, with regular or India (drawing) ink. They contain only a small amount of the ink in question, which additionally has penetrated into the structure of the paper. Extraction of ink from the substrate damages the document, and thus requires approval of the judicial body requesting the examination. Therefore, it is necessary to examine the ink directly on the document, without separating it from the substrate (paper), using, for example, infrared (IR) spectrometry and Raman spectrometry. In the obtained spectra, absorption bands originating both from ink and paper are then visible. Obtaining information about components of the ink (or India ink) requires correct interpretation of spectral data.

Materials submitted for chemical analysis rarely form a homogenous system. They are usually complex, and particular components are intermixed. In all such cases, it is necessary to separate out one or several significant components from the system and to reject large quantities of ballast material that has no significance for the case.

It often happens that the main components or fractions of the studied material are not of interest to forensic investigators, whereas particles of material attached to cracks on the surface of larger objects and probably linked with the event are significant. An example is the study of material collected from the site of a fire, constituting a mix of products of burning from all sorts of materials. The purpose of their analysis is to find traces of the agent used to start the fire, usually highly flammable liquid, which might, for example, be occluded in the pores of burnt polymer material (flooring, carpets).

Materials that are the subject of criminalistic examinations constitute material evidence in court cases. Their identification is carried out by comparing them with a reference or with so-called comparative material provided for study.

11.1.3.3 Usefulness of Microtraces for Examination

In spite of their durability, the usefulness of microtraces for research can be negatively affected by many factors. One group of factors define the objective state of microtraces at the moment of their formation at the scene of the incident (e.g. heterogeneity) and non-representativeness of particles separated from the larger whole. An example is traces of earth disclosed on clothing in the form of mud. Only a tiny fraction of soil (dust) forms the trace because only such a small sample can stay on the fabric surface.

Other factors act (on the microtrace) in the period from the moment of formation to the moment of securing the microtraces. These include secondary changes, such as ongoing biological decomposition and corrosion, and contamination by dust floating in the atmosphere. Biological material (e.g. fragment of skin containing a gunshot wound) easily degrades, making it impossible to search for traces (e.g. gunshot residue on a gunshot wound). Similarly, the progressive process of corrosion of a metal staple makes it impossible for investigators to see traces of the action of a tool (e.g. a saw blade) on its surface.

Mention should also be made of factors acting in the period between securing a microtrace and examining it, such as environmental influences (humidity), incorrect method of securing and accidental interference. For example, badly stored wet clothes easily become covered with mould, making it difficult or even impossible to look for fibres, particles of paint, and so on on their surface. The action of unfavourable factors is usually compounded by the passage of time.

11.1.4 Research Methods

To identify materials forming a criminalistic trace, it is necessary to study their morphology, determine their chemical composition (mainly qualitative) and study some physico-chemical properties. Because the sample of material forming the trace is small, microanalytical methods are applied for identification. Methods that

do not damage the studied sample or use it to a minimal degree (enabling repeated analysis by the same or a different method) are mainly applied. Instrumental methods currently play the most important role in the examination of traces. They enable results to be obtained quickly and are characterised by high sensitivity and low limits of detection for the analysed component. The results of examinations are then compared against an appropriate database (a collection of results obtained for standard/reference substances) and usually lead to group identification of the studied material. The applied methods are characterised by a high power of discrimination and thus enable differentiation of samples.

The basic principle that applies when conducting examinations is that of cross-checking. A result obtained by one method should be confirmed by other techniques. Trace examinations have an interdisciplinary character. Identification of submitted material requires the cooperation of specialists from various branches of science.

11.1.4.1 Microanalytical Techniques

Until recently, many traces, even those perceptible to the naked eye at the scene of the incident, were not secured because sufficiently accurate methods of analysing them did not exist. It was not until the development of microanalytical methods and their introduction into practice by forensic experts that analysis of traces and their use as a valuable source of information about an incident was possible (i.e. about the course of the event and the persons taking part in it).

The size of a sample forming a criminalistic trace is small, so techniques applied in its analysis should be non-destructive, enabling sample preservation or recovery after performed analyses. All optical microscopy and spectrometric techniques are such methods. Chromatographic techniques, which use the studied sample to a very small extent, are also admitted in the examination of criminalistic traces.

Microscopy

The basic method for analysing criminalistic traces is microscopy. A cycle of analyses always starts with microscopy and the obtained results determine the choice of successive examination methods. The fundamental aim of carrying out microscopic analyses is to observe the morphology of the sample, define its structure, thickness and uniformity. In the course of analyses, it is possible to disclose possible inclusions. Sometimes microscopic examination allows identification of a sample (e.g. fibres of natural origin, minerals, gunshot residues) or contamination (e.g. soil). Microscopic examination is usually used in the comparison of samples. Obtaining a similar microscopic image for two samples constitutes a premise for making inferences about their similarity and suggests a common origin. Microscopic examinations are non-destructive; even when it is necessary to

prepare microscopic preparations, a sample can always be recovered for further examination. There are two types, optical and electron microscopy.

Optical microscopy enables observation of a sample at magnifications ranging from a few times to 1000 times. The source of illumination of the sample in optical microscopy is white light, which passes through the studied sample (transmitted light microscopy) or is reflected from its surface (reflected light microscopy). Polarised light can be applied (polarised light microscopy) or fluorescence can be induced in a sample by illuminating it with short wavelength light (fluorescence microscopy).

In electron microscopes, a completely different source of energy is used for illumination – accelerated electrons. Images of surfaces obtained using electron microscopy are characterised by very good resolution and depth of field, which cannot be achieved with optical microscopes. In the electron microscope, the beam of "radiation" does not form an image in a direct way, but serves only to excite the sample. The image is formed on the basis of analysis of results of the collision of the stream of electrons with the surface of the studied object. The diameter of the beam striking the specimen is always small, of the order of hundredths of a micrometre. Electrons do not penetrate through the sample, which is why the obtained images constitute a reflection of the topography or composition of the surface layer of the studied object.

Microspectrometry

Microspectrometry is an indispensable technique in criminalistic analyses, being a combination of optical microscopy and spectrometry. Microscopy creates, records and interprets magnified images, whereas spectrometry uses emission, absorption and reflection of radiant energy by matter to determine its structure, properties and composition. On the basis of the type of energy applied, microspectrometry can be divided into IR, visual and ultraviolet (UV-vis), and Raman microspectrometry. This group also includes X-ray microspectrometry, in which an electron microscope takes the place of an optical microscope. Infrared and Raman microspectrometry enable determination and comparison of the chemical composition of studied samples; UV-vis microspectrometry serves to compare the colour of samples in an objective way that is independent of the observer; and X-ray microspectrometry allows determination of the elemental composition.

Fundamental advantages of microspectrometry are that extremely small quantities of a sample can be analysed, often without the necessity of separating it from the substrate; there is no burdensome process of preparing a sample for analysis; and multiple repetitions of measurements can be performed without destroying the sample. A further, specific, advantage of microspectrometry is the possibility of photographing and archiving the measured areas of the sample. However, a fundamental disadvantage is the fact that, in principle, microspectrometry only allows point analysis, and that is why non-uniformity of a sample and its contamination can significantly influence the results of spectrometric measurements.

The advantages of microspectrometry mean that it is now used in most criminalistic laboratories for examination of traces disclosed at the scene of an event.

Infrared Microspectrometry

The apparatus used for IR microscopy is a Fourier-transform infrared (FTIR) spectrometer coupled on-line with an optical microscope. The microscope serves to observe the sample in white light at significant magnification for the purpose of determining its morphology, as well as to select the area for analysis. The spectrometer, on the other hand, enables study of the sample by transmission or reflection measurement for the purpose of determining the chemical composition. It also provides information about the microstructure and optical properties (orientation) of the sample. It is possible to apply polarised light both in the observation of the sample and in spectrometric measurements.

In order to obtain a good quality spectrum, it is essential that a large amount of IR radiation energy should reach the detector, and also that the area of the sample analysed spectrometrically should be accurately defined. Failure to meet these conditions leads to reduced signal-to-noise ratio. This also happens in the case of non-uniform illumination of the field of vision and measurement. It is easy to demarcate the area of the sample that is of interest to the analyst using a beam of white light. The area to be analysed spectrometrically is larger as a result of diffraction of IR rays. Therefore, in the case of small samples, exact demarcation of the area for spectrometric analysis is crucial in obtaining a good IR spectrum.

This method is particularly useful for analysis of the qualitative composition of trace amounts of various substances secured as material evidence in court cases, analysis of the homogeneity of a sample, identification of inclusions and contaminations on a surface, and detection of defects in a structure. Its main drawback is the fact that the physical nature of the microsample can affect the photometric accuracy of measurement and cause distortion of the obtained spectra.

Infrared microspectrometry (FTIR) is most frequently applied for identification of microtraces such as particles of paint, plastic, fibre, rubber and glue, as well as for analysis of the chemical composition of, for example, ink or toner.

Microspectrometry in the Visible and Ultraviolet Range

This method allows comparison of the colour of very small samples of various materials (e.g. individual fibres, particles of paint, traces of ink or ballpoint pen ink on a forged document) in an objective way that is independent of the observer (i.e. the acuity and quality of his/her vision). The apparatus consists of an optical microscope with a spectrometer for analysis in the UV-vis range via an analogue digital converter with a computer. The method enables information to be obtained about the spectral differences existing between two samples of similar colour, which are indistinguishable using a comparative optical microscope. Obtaining fully consistent spectra for the compared samples attests to consistent colour and, thus, consistent pigment/dye composition of samples. Additionally, applying

appropriate software for analysis of microspectrometric results allows accurate definition of the colour by assigning a numerical value to it, the chromaticity coordinates. These coordinates ascribe to each colour a point in colour space, determined by three components describing colour (i.e. hue, brightness and saturation). Overlapping of points in colour space attests to the identical colour of samples. Measurement and description of colour were carefully standardised by the International Commission on Illumination (Comission Internationale de L'Eclairage, CIE) for the first time in 1931 [2]. Digital colour description and calculation of chromaticity coordinates were proposed about 40 years later.

Raman Microspectrometry

Raman microspectrometry is a complementary method to IR microspectrometry. An optical microscope coupled to a spectrometer enables measurement of radiation scattered by a sample with a diameter of several micrometres. Depending on the type of laser used to excite the sample, information about different components is obtained. Advantages of the method include good sensitivity and spectral resolution, very short duration of measurement, high power of discrimination and the possibility of spatial imaging of selected components within the sample. These advantages have aroused the interest of forensic chemists, among others. Most frequently, the method is used for analysis of the pigment composition of traces in the form of paint particles, individual fibres and inks on a document. As a method that is non-invasive and non-destructive (of the studied sample), it is the basic technique for examination of the authenticity of documents, enabling differentiation of inks and ballpoint pen inks directly on the questioned document. However, sometimes samples exhibit fluorescence and then the application of several excitation lasers to obtain a readable Raman spectrum (or the use of surface-enhanced resonance Raman scattering, SERRS) is required.

X-Ray Microspectrometry

X-ray microanalysis is performed using a scanning electron microscope coupled with an energy dispersive X-ray detection system (SEM-EDX) or using an X-ray microfluorescence spectrometer (μ-XRF). Characteristic X-ray radiation is emitted from the studied material as a result of bombarding the surface of the sample with a beam of accelerated electrons emitted by a cathode (in the electron microscope), or with X-ray radiation arising in the X-ray tube of the XRF spectrometer during excitation of the atoms of the anode target with a stream of electrons. Detection of this radiation, and determination of its intensity, provide information about the elemental composition of the analysed sample.

The greater the energy of the electrons of the beam and the smaller the mean atomic number of the elements making up the studied sample, the greater the depth of penetration of electrons into the sample. In the electron microscope, the penetration varies from a few tenths to several micrometres, and in μ-XRF it is significantly greater, even of the order of millimetres.

Thanks to the linear relationship between the intensity of the characteristic X-ray radiation generated in the sample by electrons and the concentration of the given element, quantitative elemental analysis is also possible. X-ray microanalysis performed using SEM-EDX is, in principle, point analysis and is suitable for studying very small samples of solid materials that are stable in an electron beam. The X-ray fluorescence method, on the other hand, can be applied to the study of both solids and liquids. The signal reaching the detector always originates from a certain sample volume, and thus it is not point analysis. It is more sensitive than the SEM-EDX method.

Pyrolysis Gas Chromatography

Among other microanalysis techniques, it is worth mentioning pyrolysis gas chromatography. The apparatus set consists of a pyrolyser, where the breakdown of the studied sample to simple volatile substances occurs, and a gas chromatograph coupled with a mass spectrometer for separation and identification of the volatile substances. In contrast to microspectrometry, this technique is considered destructive (of the studied sample) to a small extent. It is an indispensable technique for studying the chemical composition of macromolecular materials (polymers, plastics, rubber). The breaking of chemical bonds that occurs during pyrolysis of the studied sample under the influence of temperature or electromagnetic radiation in an inert gas atmosphere leads to degradation of the sample and to creation of stable fragments that are characteristic for it. Their separation on a chromatographic column and identification of particular compounds by MS gives information about the composition of the starting sample. Selection of pyrolysis conditions allows control of sample fragmentation and the formation of defined particles, which enable samples of a similar chemical composition to be distinguished (e.g. samples belonging to the same chemical class). By maintaining the same pyrolysis conditions and stable measurement conditions, one can obtain, in a repeatable way, the same type of fragment from the same starting sample. For improvement of detection of some compounds, it is beneficial to carry out preliminary derivatisation of the sample in an on-line system with the help of an appropriately selected reagent.

An advantage of this method is the fact that the amount of sample needed for analysis is of the order of 3–5 μg, depending on the type of polymer in the sample and the type of applied instrument. Its accuracy varies in the range 10–20 %. It is used in the study of traces of polymer materials such as paints, plastics, rubbers, glues and adhesive tapes.

11.1.5 Interpretation of Results

The most difficult stage of the whole process of identification of materials making up the criminalistic trace is interpretation of the obtained results of analyses, taking into account the fact that each measurement result is burdened with error. The most important thing is for the obtained results to be repeatable. Therefore, the precision of measurements must be high and the results should not be burdened with systematic errors, but close to the true values. The applied method should be accurate and reliable. Each measurement of a quantity is repeated several times, the scatter of the results observed and the measurement error determined. Practically each applied measurement method must be validated.

When processing results, simple statistical methods as well as more complex chemometric methods are used. Significance tests are applied to assess measurement results obtained for two compared samples and to establish whether small observed differences between them are the result of real differences in measured values, or whether they are the result of accidental errors.

Chemometric methods such as analysis of correlation coefficients, cluster analysis or neural network analysis are used, for example, in the classification of fragments of glass on the basis of their elemental composition or refractive index. Such methods allow the test material to be classified into the appropriate group of products on the basis of the measured parameter.

Criminalistic interpretation of the results of examinations is an important issue. Results of chemical examinations of materials constituting material evidence in a given case are helpful in identification of the perpetrator on the basis of traces. A forensic chemist thus seeks to use reliable methods and research procedures to obtain accurate data on studied samples. All the methods applied in the identification of traces allow determination of their most characteristic features (i.e. composition and properties). However, ascertaining the consistency of chemical composition and properties of studied materials is insufficient to state that they are identical. Knowledge is necessary about differentiation of the studied type of materials, variability within type resulting from non-compliance with technological norms, application and prevalence in the world around us. Knowledge of the circumstances of the course of the incident itself is also useful. That is why databases of defined types of materials that form criminalistic traces secured for examination at the scene of an incident (databases of paints, glass, plastics and fibres) are being created in individual criminalistic laboratories. Such databases contain both technological information about the products and the results of their laboratory examination.

Processing of research results leads to determination of the following conclusion: if as a result of conducted comparative physicochemical analyses, the properties and chemical composition of material forming the evidence trace are found to be consistent with those of the reference material, then, on this basis, the materials could have a common origin. This means, in the case of analysis of samples of paint, glass, plastic and fibres, that they could have constituted a single entity before

the incident. In the case of traces of soil, it means that they could have originated from one place in a given area. Categorical determination is not possible, because there is a finite but small probability that the studied materials originate from two different products, belonging albeit to the same type but, for example, from two production batches. Thus, their chemical compositions only differ insignificantly. On the other hand, if a difference is demonstrated in properties or composition of the compared materials, then one can assume that the studied materials are significantly different.

11.1.6 Examination of Chosen Microtraces

The most common microtraces examined in criminalistic laboratories are so-called contact traces (i.e. small fragments of paint coating, glass, single fibres, soil, writing materials). Moreover, traces of flammable liquids originating from fire debris or traces pointing to the use of firearms are revealed and identified.

11.1.6.1 Paint

Paint traces are revealed most often in connection with events such as car accidents, robberies or burglaries. They occur in the form of microfragments of paint, frequently with an area of several square millimetres or less, or visible smears of paint in the form of coloured streaks found on the clothing of persons involved in these events or on other substrates. The aim of paint examination is to establish the degree of similarity between the sample forming the paint trace and the sample originating from the suspect (from his vehicle, tools used in the act, etc.). Identification analysis is also carried out to determine the type of paint product, its use, the producer and the year of production. Routine examination of the paint encompasses establishing the colour and shade of the sample, the structure of the paint fragment and analysis of the chemical composition [3].

Most often, fragments of paint have a multilayer structure. Each layer (about 10–50 μm thick) is made up of paint material and is a mixture of many chemical compounds. Paint smears, on the other hand, are usually made up of one or two layers of paint material mixed with and sunk into the base (e.g. among fibres of the fabric). The morphology of the paint trace can be observed under an optical microscope. The number of layers visible on a cross-section of paint chip, their colour and thickness are characteristic for the coat of paint from which it originates (Fig. 11.1). The layers are often better visible if the sample is illuminated with polarised light or if fluorescence of sample is excited by illumination with UV light.

Colour is one of the most characteristic features of paint samples. It can be precisely described by comparison with the colour of standard paint samples from a catalogue prepared by paint producers. In the case of automobile paints, a good fit between the colour of the examined sample and the colour of a sample from a

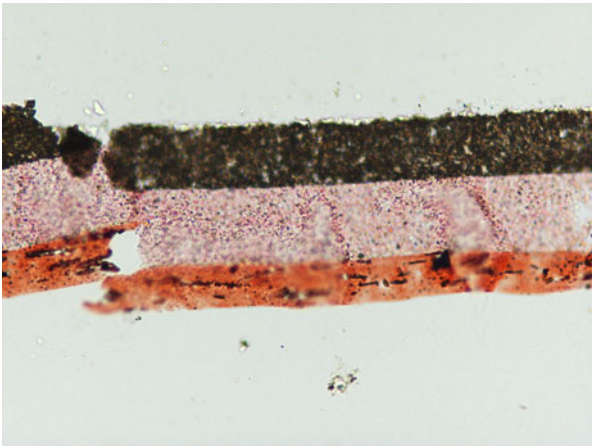

Fig. 11.1 Cross-section of a car paint chip: microscopic image in transmitted light

catalogue enables definition of the model of the car and its maker. However, visual colour comparison is subjective and depends on proper sample illumination and the sharpness and quality of the vision of the observer. Microspectrometry in the visible range (MSP-vis technique) allows comparison of the colour of very small samples of various materials in an objective way, independently of the observer, without delving into the pigment composition of the analysed samples. Each colour can be described using three variables (hue, brightness and saturation), therefore each colour is represented by a single point lying in the colour space marked out by these variables. Overlapping of points in the colour space attests to the identical colour of samples. A mathematical way of establishing variables was elaborated about 40 years ago. Parameters of colour (chromaticity coordinates) defined on the basis of measured spectra serve in assessment of the similarity of the colour of studied samples. Modelling studies have determined threshold values, which are helpful in assessment of the differences and similarities in colour [4, 5].

Every paint contains binder, which is composed of synthetic resins and a combination of organic and inorganic pigments, extenders and decorative (effect) pigments. Pigments provide the coat of paint with its colour, whereas extenders are responsible for the decorative effects of a paint coat (e.g. covering and polish) and its resistance to the activity of atmospheric factors. Paints of the same colour can contain the same polymeric base but a different set of pigments and extenders, which depend on the use of the paint and the producer of the article. The composition of the polymer binder is routinely established using IR spectroscopy. Application of GC-MS for analysis of gaseous products of paint samples enables differentiation between resins (polymeric binders) belonging to the same chemical group. An example is shown in Figs. 11.2 and 11.3. Three paint samples contain the same type of polymer binder (styrene-acrylic-urethane binder) and the same main inorganic pigment (titanium dioxide). Their IR spectra are very similar, whereas their pyrograms are clearly different. This indicates that the polymer contents of the

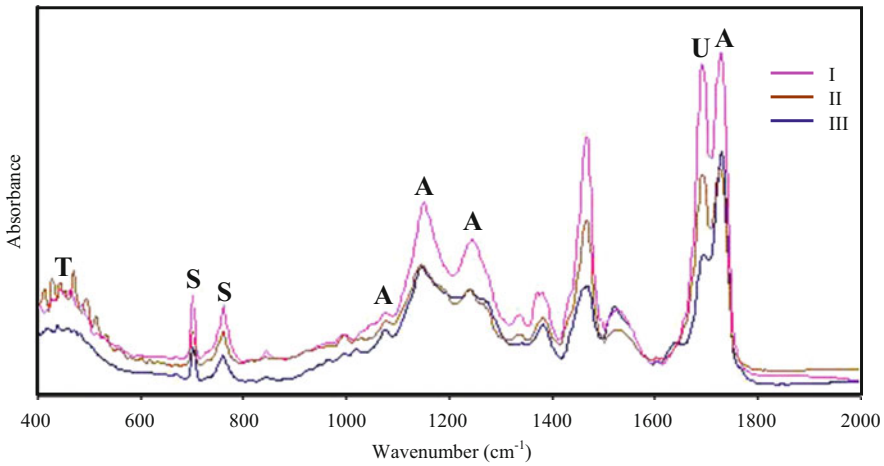

Fig. 11.2 Infrared spectra of three styrene acrylic urethane paints (*I*, *II*, *III*). *S* styrene, *A* acrylic resin, *U* urethane resin, *T* titanium white

paint samples differ significantly and that there are, in fact, three different paints [6–10].

Inorganic pigments and extenders are usually identified on the basis of the elemental composition of the paint sample, established using SEM-EDX or XRF methods. The identification of pigment content is possible based on the elemental composition of a paint sample and data on possible pigment sets used in the paint industry. Organic pigments added to paint in a very small amount can be found only with the use of Raman spectroscopy. Comparison of the obtained Raman spectra with the spectra of standard pigments from a library provides identification.

The obtained analytical data allow a conclusion to be drawn about whether the compared paint samples could have originated from the same coat of paint or not. If the reference material (i.e. from the suspect) is not available, only establishment of the kind of paint and the type of paint coat is possible. In the case of automobile paints, it is also possible to find the model of car involved in an accident. For this purpose, analytical data are compared with a database containing information about the type of paint coatings (layering, chemical composition of each layer) used in various types and models of vehicles in Europe. Such a collection has existed in Europe since 1995 and is updated every year with information on new products. Experts from many criminalistic laboratories have participated in its creation. Comparison with such a database provides information about the make and model of the vehicle involved in the incident and its year of production. It is thus helpful in identifying the perpetrator's vehicle. It should be emphasised that identifying the make and year of production of a vehicle on the basis of a paint database only applies to vehicles with a factory-new coat of paint.

There is usually only a small amount of paint visible on clothing or other substrate. So, the amount of information about the coat of paint from which it

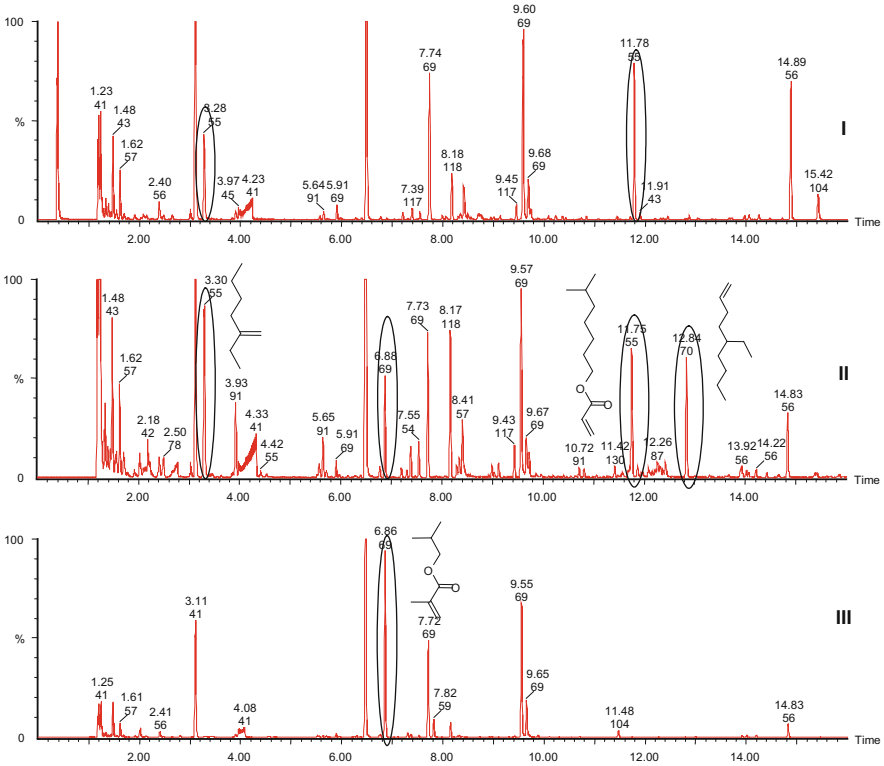

Fig. 11.3 Pyrograms of three styrene acrylic urethane paints (*I*, *II*, *III*). The differences are marked with *ellipses*

originates is smaller than in the case of a paint chip. The evidential value of it is, therefore, smaller than that of a paint chip.

11.1.6.2 Glass Microtraces

Glass fragments are known to transfer to the clothing of a person breaking a window (e.g. in a house, shop or car). Traces in the form of glass fragments are also revealed in cases of traffic accidents, fights, robberies and shots. Small fragments of glass can dislocate several meters from the broken glass object and be kept (hidden) between fibres of clothing of the breaker for a long time. Fragments of glass have various sizes. Those found at the scene of the event are large fragments, whereas those revealed on the clothing, hair or body of persons (the glass-breaker) are small, with linear dimension less than 1 mm. Routine examination of glass fragments encompasses establishing their elemental composition and determining some physical properties, such as the refractive index [11, 12]. These data can be used by a forensic scientist for comparison of samples of glass, for

ascertaining the kind of object they could have broken off from (window, bottle, headlight) and, hence, for establishing their origin.

It is worth noting that the chemical composition and properties of glass are very similar, irrespective of the type and application of the glass. Technological advances in glass manufacture have led to less variability in physical and optical properties between products manufactured by different companies, and also to less variability between different types made by the same manufacturer. Consequently, the ability to distinguish between glass fragments (the discrimination potential) has been diminished.

The major raw materials employed for the manufacture of soda–lime–silica glasses are soda ash (Na_2CO_3), limestone (CaO) and sand (SiO_2). The other components of glass are different for different types of glass. The main elements (Na, Ca, Si and Al) are present in all glass categories at nearly the same level. The differences concern other elements originating from various additives used to improve the properties of the glass or in connection with its later application, or originating from impurities in raw materials used in the production process. Their concentration is significantly lower (at trace level).

The chemical composition of glass can be determined by many methods [13, 14]. Forensic scientists prefer non-destructive methods, allowing the sample to be examined using two or more analytical methods. Another desirable feature is the possibility of simultaneous determination of several elements (analytes), using the smallest possible amount of studied material. In the case of analysis of glass microfragments, these requirements are fulfilled by SEM-EDX and XRF methods. Other instrumental techniques such as inductively coupled plasma–mass spectrometry (ICP-MS) and its modification, laser ablation (LA) ICP-MS) [8, 9], are especially valuable. They enable evaporation of a glass sample using laser and give quantitative data on elemental content. These techniques enable elucidation of more than 30 traces of elements in glass samples.

The measurement of major (by SEM-EDS), minor and trace (by ICP-MS) elements is very important for discrimination and classification of samples into glass types. It is usually helpful to be able to classify the glass into a category such as sheet, container, vehicle window, vehicle headlamp or tableware. However, it is also necessary to apply statistical methods in the characterisation of glass evidence according to its elemental composition.

A thermo-immersion method is used for the measurement of the refractive index of glass fragments. It makes use of the change in the refractive index of immersion oil with temperature. Oil containing an immersed glass fragment is heated to a temperature at which the (observed) edges of the glass fragment disappear (i.e. up to the moment when the refractive indexes of glass and liquid are the same). The glass fragment in the immersion liquid on the microscopic slide is generally put directly on the heating stage of the microscope. Measurement of the refractive index is determined by a refractometer.

Means of refractive indexes of various glass samples differ only slightly. Therefore, various statistical methods are used for comparison of glass samples when differences in their refractive indexes are being evaluated. Such methods enable

evaluation of whether the observed differences are a result of instability of equipment, non-homogeneity of glass or different origin of samples. The comparative analysis of glass microtraces on the basis of their refractive indexes and elemental composition requires the application of statistical methods to evaluate the significance of observed differences and decide whether examined samples could originate from the same glass object.

11.1.6.3 Fibres

Fibres are valuable criminalistic evidence. Fibres are a few millimetres long and loosely connected with the surface of clothing, curtains, rugs or furniture coverings. Every mutual contact between two people is accompanied by transfer of microfibres from clothing of one person to clothing of the other. Fibres are revealed mostly on clothing of people taking part in such events as murder, robbery or a fight. They are also found on underwear or under the nails of a rape victim. Fibres are revealed on the edges of an obstacle that was forced by the perpetrator (i.e. window, door or fence) as well as on tools used in the act (e.g. knife). During examination of a car accident, they could be found on a safety belt or seat covers and are useful in determination of the car driver. Moreover, recovery on the car body or chassis of fibres that are consistent with fibres of the victim's clothing confirms contact between the victim and a car.

Fibres are collected during optical examination of evidence [15, 16]. The aim of their examination is classification of fibre type and establishment of the type of textile from which they originate. During examination, chemical composition and some physical properties are established. The methods applied are optical microscopy, spectrometry in the UV-vis-IR range and Raman spectroscopy. Optical microscopy in transmission and reflection mode provides information on morphology as well as the shape of cross-section and thickness (Fig. 11.4). If polarised light is applied, the crystallinity can also be observed. The information obtained enables ascription of the fibre to one of the main types. Some synthetic fibres such as polyamides (PAs) and polyethylenes (PEs) have a very characteristic appearance in polarised light; therefore, it is possible to differentiate between fibres of similar colour and morphology observed in white light. The presence of fluorescence, originating from pigments, additives and detergents, is also helpful in identification

Fig. 11.4 Microscopic images of common fibres: (**a**) cotton, (**b**) wool and (**c**) acrylic

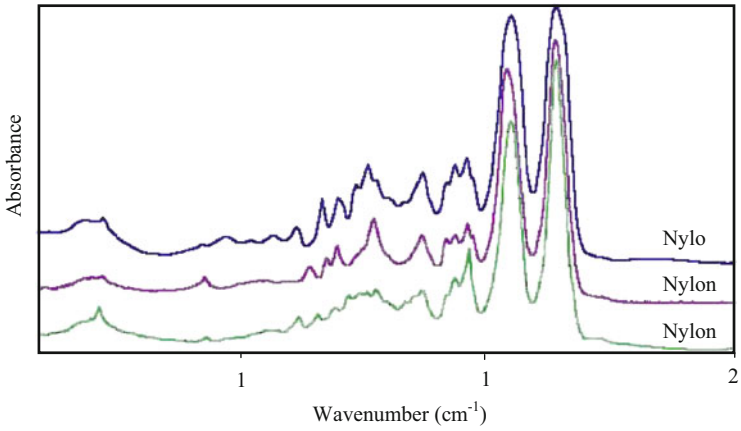

Fig. 11.5 Infrared spectra of different polyamide fibres

of fibres. Generally, it is possible to differentiate between natural and synthetic fibres, to identify some kinds of samples and to determine the distribution of pigments and additives in fibres. The main components of fibres are natural (e.g. cellulose, casein, keratin) or synthetic (e.g. PA, PE, polyacrylonitrile, polyolefins) polymers. They can be identified using IR spectroscopy (Fig. 11.5). The readable spectrum of single short microfibres can be obtained using microscopy or the diamond cell technique. It is possible to differentiate between polymers within the same chemical group; for example, different polyamides have different spectra in the range 1000–15,000 cm^{-1}. Normally, pigments and dyes are not visible in the IR spectrum of a fibre. For their identification, Raman spectroscopy is applied [9] and the spectra clearly show peaks originating from pigments, dyes and extenders.

For comparison of the colour and shade of fibres, UV-vis microspectrometry is routinely applied. Consistent spectra obtained for compared fibres confirm consistent pigment content; however, the pigment and dye set are not identified. Black and colourless fibres are exceptions because they have no colour (i.e. the most characteristic feature of a fibre) and so cannot be compared in this way.

Information about the type of fibre and its components helps in comparative analyses that aim to state similarity between transferred fibres and fibres of the suspect's clothing. The identity of analysed features provides the conclusion that the fibres originate from the same fabric, which means that fibres from the clothing of one person were transferred to the clothing of another. The evidential value of revealed fibres is different and depends on the kind of fibres. The most prevalent fibres (i.e. white cotton) have the smallest evidential value. They originate from underwear, bed linen or dust and so their recovery does not mean that they were transferred from the clothing of the suspect. Similarly, the value of jeans fibres is small because of the popularity of jeans clothing. The significance of fibres that are seldom met in the environment is greater.

11.1.6.4 Gunshot Residue

Examination of gunshot residue (GSR) plays an important role in establishing some circumstances of a crime involving use of a firearm. This kind of examination is complementary to ballistic examination of weapons and ammunition.

Powder gases leaving the barrel of a firearm contain products of explosive reactions of both the primer and the propellant, as well as products of interactions of these materials with other parts of the cartridge and weapon. The chemical composition and properties of GSR depend directly on the kinds of materials used in production of the ammunition. The most characteristic GSRs are metallic particles arising from components of the primer, demonstrating characteristic morphology (size of the order of micrometers, approximately spherical shape) and specific chemical composition (lead, antimony and barium in the case of lead ammunition) (Fig. 11.6). Found around the gunshot hole and on the clothing and body of the shooter, GSR provides information on which to base, among other things, inferences about the shooting distance and the kind of ammunition used (and thus the weapon). Most importantly, GSR serves to link the suspect to the shooting [17–21].

Micron-sized particles, having a morphology consistent with rapid cooling, form a liquid state. Such GSR particles contain elemental combinations of either lead/barium/antimony or barium/antimony and are unique to detonation of the primer of a round of ammunition.

For many years there was no sufficiently specific method for the identification of characteristic GSRs. One could not see metallic particles because of their small size (5–50 μm) and their presence was ascertained indirectly by means of colouring chemical reactions or such instrumental methods as atomic absorption spectroscopy (AAS), neutron activation analysis (NAA) or XRF. These methods, however, are

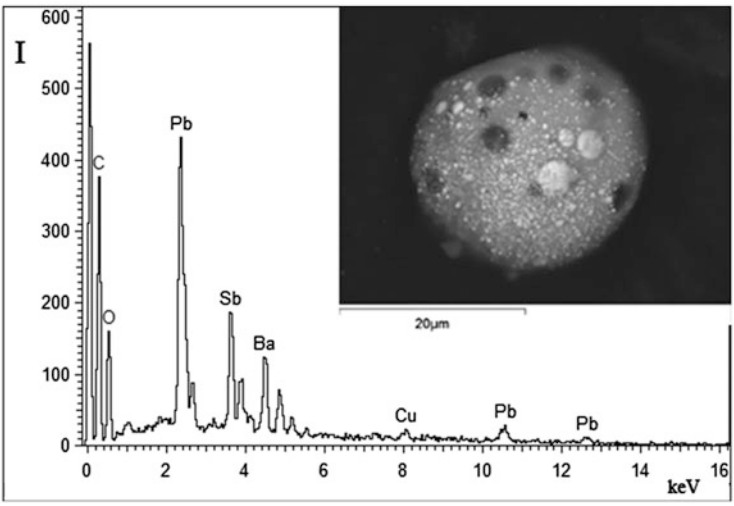

Fig. 11.6 Gunshot residue particle (*inset*) and its elemental composition

not specific and record all impurities, independent of their source of origin. The most successful technique to date for the analysis of GSR particles is, without doubt, SEM-EDX [17].

Only a little sample preparation is needed. Mostly, half-inch diameter aluminium stubs with an adhesive layer of double-sided tape are used for sampling. When the sample has been secured on the stub, it is then examined for spherical metallic particles of defined diameter and chemical composition. The method has many advantages, but its basic drawback is that it is time-consuming if carried out manually. Suitable software for automatically searching through the secured material on the stub (in order to detect particles with specific features) shortens the time of investigation significantly [17].

According to the formal approach to evaluation of analytical results, metallic particle classification can be carried out in the following way: the most characteristic particles (Pb, Sb, Ba) are singled out, followed by indicative one- and two-component particles (e.g. antimony, lead, lead/antimony), which always accompany the former and occur in considerably greater quantities. Finding GSR on material received from a suspect confirms his or her participation in the event, meaning that the person was shooting, present in the near vicinity of a firing gun or came into contact with an object highly contaminated with GSR.

Because the technology of ammunition production has changed towards leadless solutions, the evidential value of GSR should be individually established for every case in a case-by-case approach. For this, knowledge of the persistence and prevalence of GSR in certain environments is crucial [17, 18].

Solid particles of material other than metal, such as powder grains and the products of their conversion, found on targets not far from the gun can be identified from their IR spectra, taking into account the main composition of smokeless propellants (i.e. nitrocellulose or nitroglycerine). Components occurring in smaller amounts (e.g. diphenylamine or centralites) can be identified by means of Raman spectrometry. This information can be used to determine the shooting distance, to recognize the entrance and exit gunshot holes, as well as for differentiation between residues from various types of ammunition [18].

Volatile and gaseous products of propellant combustion remain inside the gun barrel and cartridge case for some time and their detection and quantitative determination by means of gas and liquid chromatographic methods (thermal desorption GC-MS, GC-thermal energy analysis, LC-MS/MS) can help in establishing, whether the gun was fired up to 3 days, 2–3 weeks or more than 3 weeks before examination [19, 20].

11.1.6.5 Fire Debris

The identification of various flammable liquids that can be used to start a fire is one of the tasks of the forensic laboratory. These products (usually petroleum products) include gasoline, fuel oil, lubricant oils and diluents. They are often used by perpetrators because of their physico-chemical properties, such as volatility and flammability.

The spilled flammable liquids sink into the base (e.g. soil, textile, wood). The burning process only takes place on the surface of the base. Deep down, liquids hardly evaporate and burn very slowly; some are occluded in porous materials created from synthetic materials during the fire. That is why it is possible to find traces of flammable liquids in fire debris in spite of them being burnt out of almost everything [23, 22].

The fire scene is carefully examined after a fire has been extinguished. If arson is suspected, samples for laboratory analysis are collected from the place that is considered to be the probable source of the fire. Usually, such examinations are limited to a search for traces of flammable liquids, which are commonly used as accelerants. Although the problem of analysis of fire debris is not a new issue, detection and identification of flammable liquids still remains a challenge. Difficulties arise because there are many different commercially available agents that can be used to light fires, most of which are multicomponent mixtures. They contain hydrocarbons (petroleum products) mixed with other solvents and flammable liquids (e.g. ether, alcohol, turpentine) and condensed by adding substances such as resins or plastics. Mostly, liquids such as benzene, kerosene, motor oil, alcohols and solvents are present. In the course of a fire, compounds contained in these mixtures evaporate to different extents, and some of them undergo thermal decomposition [22]. Burnt materials could also undergo pyrolysis, which results in the presence of numerous interferents in the sample, making analysis more difficult. Therefore, accelerants present in a sample of fire debris are available only in trace amounts and, moreover, their chemical composition significantly differs from the composition of the unburnt liquids An additional factor that can influence the effectiveness of detection of accelerants is the use of various fire-fighting agents, which introduce additional substances to the analysed material.

The main method applied in analysis of fire debris is GC. The analysis of fire debris has three stages. The first stage is isolation of accelerants from the matrix and their concentration, followed by separation of particular components and their chromatographic analysis and, last, identification of potential accelerants. The efficiency of the first stage strongly determines the possibility of identification of the isolated and adsorbed organic compounds. An improperly performed first stage could make it impossible to identify the questioned substances.

The isolation and concentration of petroleum products can be performed in several ways. The most efficient method is passive adsorption. In this method, the sample along with a tube filled with Tenax TA adsorbent is placed in a thermostated (60–70 °C) tightly closed container, such as a glass jar, for over 10 h. Under these conditions, a balance between compounds present in the headspace of the sample and the sample adsorbed on the polymer adsorbent is established. Adsorbed compounds are subjected to thermodesorbtion; then, the desorbed compounds together with the carrier gas are injected onto a GC column, where they are separated and then identified. This approach has enabled easy detection and identification of trace amounts of petroleum products. Headspace analysis with passive adsorption on Tenax TA is normally used for separation and concentration of analytes. Gas chromatography coupled with an autothermal desorber and a mass spectrometer (ATD-GC-MS) is the best technique for separation of multicomponent mixtures

and identification of components in the analysed product. GC-MS enables qualitative and quantitative analysis of traces of flammable liquids.

Interpretation of the obtained chromatograms must take into account the changes that flammable liquids undergo during a fire. One change is evaporation of light volatile compounds from the examined material, causing a decrease in the content of volatile components and an increase in content of components of lower volatility. The second change is thermal breakdown of components of liquids and burnt materials. The effect of this is appearance of volatile substances that were absent in the flammable mixture at the beginning, which makes it harder to identify the liquid used to start the fire. It is also possible that these compounds were present in burnt material at the beginning and are the components of burnt material.

During experiments carried out to study whether various fire-fighting substances influence the detection of traces of accelerants in samples taken from a fire scene, it was suggested that the application of extinguishing foam is linked to introduction of substances into the samples. Although these substances might be detected in the process of analysis, their presence does not hinder identification of the accelerant, provided the person conducting the analysis knows how to interpret the obtained results correctly.

Currently applied methods enable easy recovery of trace amounts of flammable liquids, provided that samples are properly collected from the place of fire, saved in hermetic containers and sent directly to a criminalistic laboratory. Figure 11.7

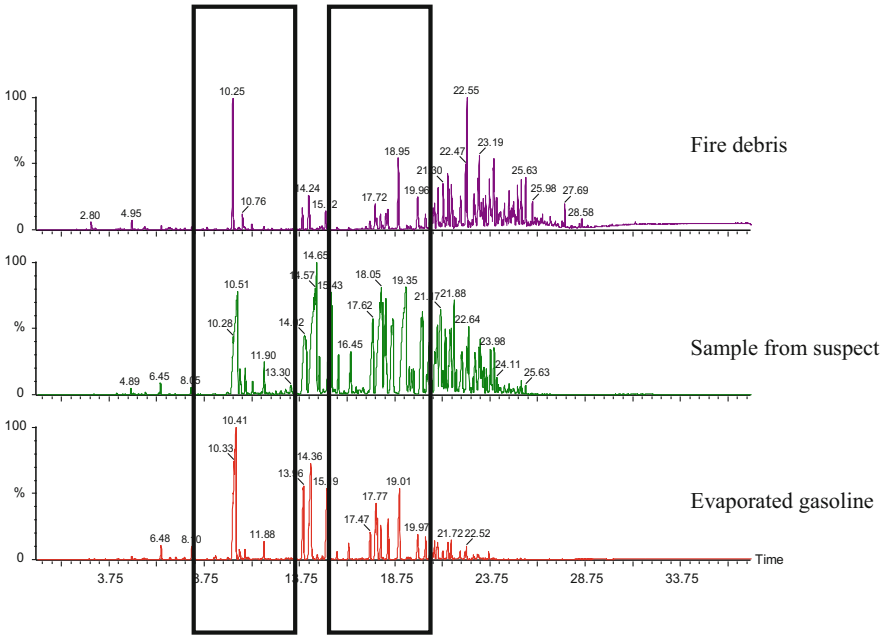

Fig. 11.7 Chromatograms of samples of fire debris, from the can found in the subject's possession and gasoline evaporated to 50 %. The characteristic patterns are marked with *rectangles*

shows the results of analysis of fire debris collected from an office. Comparative material was found at the suspect's house in an empty plastic container. Chromatograms were obtained for debris and for vapour from the recovered gasoline container. On both chromatograms, characteristic profiles consistent with the profile of gasoline evaporated by 50 % were visible. It was concluded that the flammable liquid used to start the fire was gasoline.

11.1.6.6 Writing Materials

Chemical analysis of writing materials such as inks and toners is a very important part of forensic examination of questioned documents. The investigations are most often aimed at authenticating a document or determining its age or origin. Non-destructive analytical methods, such as microscopic and optical techniques, are applied first in routine examinations of inks. Selected parameters of the ink, such as its colour, luminescence and absorption of radiation can be determined using these methods [24]. Use of transmission, reflection and fluorescence spectra in the range 220–900 nm makes it possible to differentiate samples obtained for inks, ballpoint pen inks and printing paints placed on the surface of paper in a more objective way than by comparing their colours using a tintometer.

However, such optical and spectrometric methods do not provide information on all components of ink and do not allow identification of ink on the basis of its chemical composition. They take into account only those components that strongly interact with a given region of electromagnetic radiation and, therefore, they merely show differences between compared samples. Ballpoint pen ink contains a dye or several dyes in a viscous liquid, which is a mixture of natural or synthetic polymers and an oil or olein. The ink also contains acidic compounds, which decrease its coefficient of friction during writing, and substances that inhibit drying of the paste and ensure its suitable viscosity. The detailed recipes are patented. In the literature, there is no information on the composition of gel inks. It is only known that, apart from other components, they contain insoluble pigments.

As a rule, chemical methods used in the examination of writing materials require initial preparation of a sample for study. Paper chromatography, thin-layer chromatography and capillary electrophoresis are experimental techniques often applied. These methods lead primarily to separation of the dyes contained in the ink under examination and to the discrimination of ink samples. The techniques are simple to use, require a small amount of sample for examination, are selective and give reproducible results. Their basic disadvantage, however, is the necessity to isolate the ink from the substrate (e.g. paper) on which the examined document has been prepared. Solvent extraction of the ink often leads to partial damage of the document.

Spectrometric methods such as IR spectroscopy give information on the main components of the examined samples (dyes, resins and oily liquids). The main pigments are easily detectable in the IR spectra of inks. Because of its non-destructive nature, Raman spectroscopy is applied in forensic investigations for the identification of inks directly on a document, and for determination of the

sequence of handwritten lines. Attempts have been made to utilise this method to examine the process of ageing of inks. Additionally, HPLC and GC-MS, as well as quantitative elemental analysis, are used for identification of some ink components. Each method applied for ink examination has its advantages and limitations. In practice, several analytical methods are required for characterisation and identification of inks [24].

Differentiation of inks through their IR spectra is based on the position of peaks and their relative intensity. In Raman spectroscopy, the course and shape of the background curve, which depicts the fluorescence intensity of the examined material, is also relevant and should be taken into account.

The elemental composition is useful for characterisation of inorganic pigments and organic dyes. However, a database is needed for their identification. In all ballpoint pen inks, sulfur, copper, silicon and phosphorus are present in the elemental composition. Some samples also contain zinc, chlorine, bromine and calcium. In black inks, chromium and lead are additionally found. Samples differ with respect to the elemental composition quantitatively rather than the qualitatively. A greater variability in elemental content is observed for gel inks.

Toners are another type of writing material used in printers and copiers. They are black or coloured and contain grains of synthetic polymers with characteristic plastic and electrostatic features. During printing, the grains of toner are connected with the paper, but it is easy to remove them without damaging the surface of the paper. Their size, shape and composition depend on the kind of toner. Using IR spectroscopy it is possible to identify these polymers (e.g. styrene, polyethylene, acrylates and methacrylates). Elemental composition using SEM-EDX has identified iron as the main element in ferromagnetic toners (Figs. 11.8 and 11.9). In non-ferromagnetic toners, the main elements are Zn or Cr and the content of iron is smaller. The determination of polymers and elements makes it possible to classify toners into several groups and sometimes to identify the kind of printer [25–27].

11.1.7 Conclusions

In forensic examination of different materials, various analytical methods are applied. These methods should enable analysis of small amounts of samples (milligrams or micrograms) and be non-destructive, making it possible to repeat analysis using the same or different methods. The methods are quick and simple and do not require time-consuming sample preparation before analysis. They provide information about chemical composition and about some physico-chemical features of analysed samples, enabling group identification of the examined sample. Forensic chemists look for microcomponents of the examined sample or features that are distinctive and enable differentiation of the sample from other samples belonging to the same chemical group. Therefore, in forensic examination it is necessary to examine small amounts of sample and to identify trace components, because the main components do not usually characterize the examined criminalistic trace.

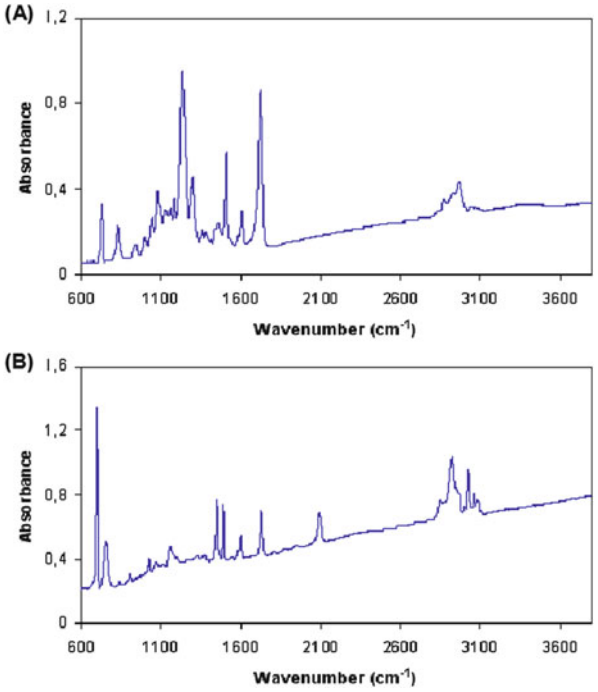

Fig. 11.8 Infrared spectra of two different toners (**a**, **b**)

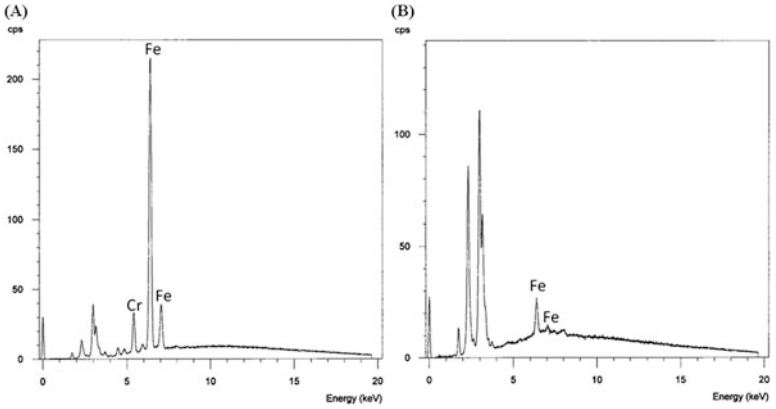

Fig. 11.9 XRF spectra of toners: (**a**) ferromagnetic toner and (**b**) non-ferromagnetic toner

Forensic chemistry applies new and sensitive analytical methods in identification of traces and collects technological information about various materials to create a database. For the evaluation of results obtained, statistical methods enable assessment of significance of differences observed between the examined samples, as well as the statistical error.

11.2 Toxicological Analysis of Microtraces

Maria Kała

11.2.1 Introduction

Toxicology is the science of poisons. It was established as a separate academic discipline, separating from forensic medicine, at the beginning of the nineteenth century. At that time it dealt only with detecting poisons in autopsy material; in other words, it assisted forensic medical doctors in issuing opinions on the cause of death in cases of poisoning. Since then, toxicology has developed in many directions and is currently an interdisciplinary science. It builds on the achievements of the basic sciences and cooperates with various applied disciplines. This has led to specific research areas being separated into areas such as the toxicology of drugs, pesticides and food; environmental, industrial, clinical and forensic toxicology; and various sub-disciplines (e.g. toxicological analytics). Modern toxicology deals with the analysis of qualitative and quantitative effects of the harmful action of chemical compounds on living organisms.

This paper presents contemporary toxicological analysis for the presence of poisons, which are increasingly frequently detected in the human organism at very low concentrations. It starts with a discussion of those elements of toxicology that are of greatest relevance to (toxicological) analyses performed at the behest of the administration of justice. Attention is paid to types and sources of poisons, the influence of the route of introduction of the chemical compound into the organism on the course of poisoning, the type of compound whose presence must be demonstrated in order to confirm or rule out exposure, the scope and directions of toxicological analysis, and research methodology.

Currently, at least 1200 scientists, members of The International Association of Forensic Toxicologists (TIAFT), work in the field of toxicological analysis for the needs of the administration of justice. Two reference books hold a key position in the work of forensic toxicologists (the issuing of expert opinions). One is the extensive monograph *Clarke's analysis of drugs and poisons* [28], bearing the name of an eminent English toxicologist and currently available in its fourth edition. The second work, *Disposition of toxic drugs and chemicals in man*, is updated every couple of years (the tenth edition is currently available) [29].

11.2.2 Types of Poisoning

The harmful action of chemical compounds, affecting humans and other forms of life, can occur everywhere (i.e. in the wider human environment, including the workplace). Poisoning can occur accidentally or as a result of deliberate human action (suicide, murder). Depending on the speed of development of the harmful

action of a toxic compound on the organism, the poisoning can be defined as acute (quick development of harmful changes after taking a single dose), sub-acute (changes occur less rapidly) or chronic (action of poison in small doses over a long period of time). From these definitions, it is clear that we are dealing with varying amounts of toxic factor. A large single dose of a toxic compound usually results in a high concentration in the organism, whereas small multiple doses lead to low concentrations of the compound and/or its metabolites (i.e. products formed in the organism as a result of biotransformation processes). Analytical methods applied to confirm or rule out the presence of a chemical substance in the organism of a human must be characterised by high accuracy, sensitivity and specificity.

11.2.3 The First Poisons

Harmful chemical compounds called poisons have accompanied humanity since ancient times. The first poisons were substances of natural origin, that is, toxins (produced by plants), venoms (produced by animals) and mineral substances (As, Sb, Sn and Cu). Dynamic advances in the synthesis of chemical compounds, initiated at the turn of the nineteenth and twentieth century, as well as the rapid progress of civilisation, led to introduction into the human environment of a huge number of synthetic chemical substances. Currently, therefore, we are faced with an increasing number of toxic substances of natural and synthetic origin.

Toxicity Since ancient times, people have been aware of the dual nature of chemical substances. Many substances are applied in low doses in the treatment of diseases, and these same substances are used as poisons in high doses. Not all chemical substances introduced into the living organism or produced in it are equally harmful. Very toxic substances cause dysfunction or death of an organism in amounts (doses) of a few drops or tens of milligrams. The lethal dose for relatively harmless or practically non-toxic substances is defined at a level equal to or greater than a kilogram. The relationship between dose and toxicity was described by the father of toxicology, Theophrastus Bombastus von Hohenheim Paracelsus, in the sixteenth century in the first definition of poison: "All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison." This definition remains true to the present day, taking into account not only the dose (administered or absorbed), but also the route of administration (oral, inhalation, intravenous or dermal) and frequency of administration (once or many times).

11.2.4 Modern Spectrum of Poisons

Factors Affecting the Course of Poisoning and Interpretation of Results of Toxicological Analysis These days it is rare to come across poisoning as a result of a single substance or high dose of low-toxicity chemical substance. Most frequently, poisonings are caused by introduction into the organism of a mixture of many substances. This leads to "interaction" effects of these substances. As a result of interaction, the action of many substances gives results that are qualitatively or quantitatively different from the predicted actions, arising from the sum of the effects brought about by the individual components of the mixture. Thus, small doses of various compounds lead to low concentrations of each separately, and can result in severe intoxication. Revealing all the components of an administered mixture in biosamples (body fluids and/or sections of internal organs) is important in assessment of the severity of poisoning and requires application of sophisticated, very sensitive, specific and selective analytical methods.

Many substances (agents) that are a threat to the health or even life of the consumer can be the subject of toxicological analysis. In view of our ability to detect smaller and smaller amounts of xenobiotics (chemical substances that are not natural components of a live organism) in the analytical process, interpretation of the obtained results assumes particular significance. Interpretation must take into account both the concentration of the detected xenobiotic and the individual sensitivity of the victim. Individual sensitivity varies according to the properties of the xenobiotic and intra-individual factors (i.e. race and sex). Furthermore, sensitivity changes with age and is linked with congenital and acquired disease states (allergies, diseases of civilisation) and genetic defects, which are more and more prevalent. Drug addiction, addiction to medication, polytoxicomania and polypragmasia (abuse of drugs that harm health), which frequently lead to dependence, tolerance (long-term drug use necessitating an increase in dose in order to induce the desired effects) and abstinence syndromes, are currently widespread. The broad use of polytherapy (treatment with several medications) has resulted in the availability of many different types of pharmaceutical drugs.

Medications In cases of poisoning by a single harmful agent or a mixture of medications and/or toxic substances, compounds of various chemical nature and numerous metabolites are present in biological material. The possibility of occurrence of various types of interaction (synergistic, additive, hyperadditive and antagonistic) at each stage of the course of poisoning (absorption of the xenobiotic, distribution in the organism, biotransformation, action and excretion) after administration of several drugs makes it much more difficult to interpret the result in terms of the severity of the poisoning and adapt remedial treatment. Independent administration of medications, without consulting a medical doctor, is noted increasingly frequently. The type and dose of substances introduced into the body are rarely known. Knowledge of the type and toxicity of the compound facilitates the choice of an appropriate method and suitable material for analysis.

Currently, substances that are characterised by many times the toxicity of compounds regarded as the greatest poisons (cyanide, As, Tl) are used in medical treatment and are thus potentially (widely) available. A striking example of the dual nature of a substance is the botulinum toxin, which is an exceptionally strong poison but finds application in medicine (for treatment of spasticity of muscles, involuntary muscle contractions and excessive sweating) and even in the cosmetic field for aesthetic purposes (i.e. temporary reduction of wrinkles). Forensic toxicologists increasingly frequently encounter an expanding assortment of drugs used for in-patient healthcare, each dose of which when introduced into the human organism without medical supervision can be fatal. Use of such drugs during an unsuccessful operation necessitates asking whether the therapeutic dose of the medicine had been exceeded.

Environmental Poisons Environmental and industrial poisons constitute another group of very toxic compounds. Among the environmental poisons are dioxins, which humans themselves produce by burning various types of waste in their homes. As a result of increasingly stringent requirements concerning safety at work, industrial poisoning most frequently occurs accidentally or as a result of negligence. In spite of the fact that acute poisoning by inorganic compounds (metals, semi-metals and anions) is becoming increasingly rare, its significance in contemporary toxicology cannot be overlooked. People living in heavily industrialised regions are more exposed to low doses of industrial and environmental poisons as a result of environmental contamination. Food, atmospheric air and water from these areas are constant sources of exposure (of inhabitants) to small doses of metals, which can accumulate in the body. Ruling out chronic poisoning, which may have occurred in conditions of environmental or industrial contamination, requires knowledge of normal levels of metals (i.e. their concentrations in the organs of non-poisoned persons). Metals can be divided into the following groups: very toxic (As, Be, Cd, Cr, Pb, Hg and Ni); essential for the correct functioning of the human organism, but characterised by a high potential for toxic action (Co, Cu, Mn, Se, and Zn); and low toxicity (Sb, Ba, In, Mg, Ag, Te, Tl, Sn, Ti, U and V). Such categorisation is useful in the assessment of exposure of humans to metals. A fourth group is made up of metals whose toxicity results from their application in therapy (Al, Bi, Ga, Au, Li and Pt). Detection of toxic anions such as nitrates(III) and nitrates(V), fluorides, oxalates, chlorates, phosphides and sulfides is also within the area of interest of toxicologists. Many of these are used as artificial fertilizers, in the household, and even in the food industry.

The Drugs (Narcotics) Market An unlimited source of substances that are the subject of contemporary toxicological analyses is the drugs (narcotics) market. It has long provided a broad range of synthetic substances (e.g. amphetamine and numerous derivatives) and substances of plant origin (e.g. marijuana, hashish, hallucinogenic mushrooms) that are under international control. Here, they are classed together under the term “classic psychoactive drugs”, enabling discussion of these agents to be kept brief. Changes in drugs available on the Polish drugs (narcotics) market over the years, from substitutes for drugs of abuse to the current

drug scene, have already been described many times [30, 31]. However, it is impossible not to mention the large group of new compounds, the so-called designer drugs, which are structural analogues of controlled substances. Among them, the following are most prominent: derivatives of phenylethylamine, cathinone, piperazine, tryptamine and the so-called synthetic cannabinoids. Many of these substances are smuggled, but they can also be produced in large illegal clandestine laboratories. Another group consists of home-made substances, for example, from medicines available without prescription [32, 33]. Many substances that are newly introduced onto the drugs market are characterised by increasingly strong action. Unpredictable mixtures of known substances have become the cause of lethal poisonings of many uninformed users [34]. In 2006, genetically modified cannabis appeared on the drugs market, containing small amounts (below 0.20 %) of δ^9-tetrahydrocannabinol (9THC) and a high content of δ^9-tetrahydrocannabinol-2-carboxylic acid (9THCA-A), which is converted to 9THC during smoking. In order to determine whether cannabis secured by the police is subject to the provisions of the Act on the Prevention of Drug Addiction (i.e. whether it is of the fibrous or narcotic variety), methods allowing determination of each compound separately (high-performance liquid chromatograph, HPLC) or both together (gas chromatography, GC) should be applied to their analysis [35].

Online Shopping Online purchases from websites remain beyond any sort of control. It is possible to put anything from anabolic steroids, through doping agents to various slimming products, especially natural products of Chinese origin, into your "basket". The latter are advertised most frequently as safe preparations of plant origin that aid slimming, but often contain large doses of synthetic compounds with the structure and activity of amphetamine derivatives. Furthermore, some Chinese herbal preparations have such a high lead content that if they are administered long term they engender symptoms of poisoning by this metal. The same applies to pesticide residues contained in these products.

New Psychoactive Substances The term "new psychoactive substances" (NPS) (legal highs, boosters) is used to describe various types of preparations or substances contained in them, which are sold as collectors' items, bath salts, agents for rinsing river stones, plant care agents, incense and the like, not intended for human consumption but in fact used for intoxication purposes. These products are often advertised as "legal alternatives to drugs". The attractive names of these substances, for example, Energy pills, Euphoric pills, Psychedelic pills, *Salvia divinorum* (Diviner's sage), Magic garden, *Amanita muscaria* (Fly agaric) and Indian warrior, under which they are available in brick-and-mortar and internet shops, and even by telephone order with home delivery (as well as assurances about their low harmfulness), have encouraged many young people to experiment with them, often ending in poisoning. Since November 2010, the psychoactive components of many of these types of products have been placed on the list of substances controlled by the Act on the Prevention of Drug Addiction. Various types of preparations were and are on sale, from powders and tablets to capsules with herbal mixtures. Laboratory studies have shown that, in most preparations, the plant

material only constituted the carrier onto which synthetic cannabinoids were deposited (JWH-type, followed by a digital symbol such as 081, 007, 018, or similar) [36]. Since 2008, the presence of more than 30 synthetic cannabinoids has been ascertained in legal-high products around the world. In the last 10 years in Europe, about 100 new substances with psychoactive activity have been discovered. According to the reports of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the largest group is phenethylamines (32 compounds), then tryptamines (22), cathinones (15) and piperazines (12) [37, 38]. The mass spectra of these new compounds, together with the spectra of various additives present in commercial preparations, have been published in a combined study [39].

Date-Rape Drugs The colloquial term "date-rape drug" encompasses about 70 different drugs or psychoactive substances that are applied to facilitate commission of a crime (rape or looting). A typical scenario of such an event consists in adding such a substance unnoticed to a drink, which the potential victim of the future crime consumes. The victim quite quickly loses awareness of what is happening, and all memory of what has happened is impaired. After regaining consciousness, amnesic symptoms are accompanied by physiological disorders such as lack of spatial orientation, dizziness, drowsiness, difficulty moving, nausea and sometimes hallucinations. Sometimes the victim sees the attacker but cannot defend him- or herself, because the administered substance has caused difficulty in moving or temporary paralysis. Generally, the victim's recall of the event is so unclear that s/he is not a credible witness for courts and other judicial bodies. In such situations, only chemico-toxicological analysis of body fluids can confirm the use of a pharmacological substance. Usually material for testing is collected after several days, and thus the applied methods must be very sensitive and encompass many compounds and their metabolites [40, 41].

Agents Acting Similarly to Alcohol Use of agents acting on the central nervous system modulates the behaviour of people and affects driving ability. That is why provisions of the Road Traffic Act forbid driving of a vehicle by a person "in a state after use of alcohol" and "in a state after use of substances acting similarly to alcohol". Drivers can be subjected to tests for the presence of these substances at the place where they are stopped by police, using methods that do not require laboratory facilities. Breathalysers are used to demonstrate the presence of alcohol in a driver's body. These devices enable determination of alcohol in exhaled air. This result has evidential value. Drivers are controlled for the presence of substances acting similarly to alcohol on the basis of observation of symptoms of action of these substances on the body of the driver, as well as by using on-site oral fluid drug screening devices. Such tests enable determination of illegal compounds from five groups (opiates, tetrahydrocannabinols, cocaine and its metabolites, amphetamines and benzodiazepines) in drivers' saliva. Each positive result of analysis using an on-site oral fluid drug screening device must be confirmed by a blood test carried out on the driver using laboratory methods such as GC or HPLC) with various types of detection, especially mass spectrometry (MS). Only then does the result of toxicological analysis constitute a basis for prosecuting a driver. The applied

laboratory methods must be characterised by limits of quantification (LOQ) imposed by the Order of the Minister of Health of 16 July 2014. All LOQ values are defined at the level of nanograms per millilitre of blood and are 1 ng/mL for 9THC, 10 ng/mL for morphine and cocaine, 25 ng/mL for amphetamine and its derivatives and 100 ng/mL for benzoylecgonine. The LOQ has not been established for drugs of the benzodiazepine group, because of the numerous derivatives applied in very diverse doses (varying from 2 to 25 mg), which result in the occurrence of a very broad range of concentrations in blood [42].

Toxins and Venoms Because human interest does not always develop in the right direction, the number of venoms and toxins that the forensic toxicologist can encounter in his or her everyday work is growing. Several hundred toxins in various species of plants, and venoms in many species of marine organisms, poisonous fish, venomous snakes, tics, scorpions and spiders are known. Furthermore, there are species of birds whose feathers can be triggered to act toxically almost by touch alone. On occasion, such exotic specimens, for example, acquired during travel abroad or bought over the internet and then bred in unsuitable conditions (and sometimes abandoned) may become the cause of laborious toxicological tests of body fluids taken from a bitten victim.

11.2.5 Routes of Introduction of Poison into the Body

A toxic agent can enter the body by various routes. The development of poisoning depends primarily on the toxicity of the substance, which is linked to the dose but also the route of administration. Intravenous xenobiotic administration results in the entire dose being quickly introduced into the organism. The toxic dose is smallest for a poison given intravenously. A dose of xenobiotic given orally is not always completely absorbed into the organism and, therefore , the administered dose is not always the acting dose. Part of the administered dose is removed as a result of natural defensive mechanisms of the organism against poisoning, for example, vomiting. A large part of the administered dose of many xenobiotics first passes from the stomach via venous blood through the portal vein to the liver, where it is metabolised, and then goes into the systemic circulation in the form of metabolites. This process is known as the first-pass effect. If the metabolites are less toxic than the administered (parent) substance, then the toxic effect is reduced. In the opposite case, when the metabolites are more toxic than the administered substance, the course of intoxication is more severe. Volatile xenobiotics (toxic gases and solvents) enter the body through the respiratory system (by inhalation) and, with air, enter the alveoli, which are richly supplied with blood and have a large surface area. A large momentary dose of xenobiotic can result in the occurrence of very rapid changes, which does not, however, translate into a high concentration in the bloodstream (and by the same token in the organism). Many poisons, such as organophosphate pesticides, are absorbed through the skin and not only have

negative health effects, but can also be a threat to life itself. Other routes of administration, such as topical, rectal and intramuscular, have great significance in medicine, but are also encountered in cases of suicidal and deliberate poisoning, for example, dowsing a victim with a corrosive or burning substance. Depending on the route of introduction of the xenobiotic into the organism, the analyst has to deal with a lower or higher concentration of xenobiotic and/or its metabolites.

11.2.6 Symptoms of the Action of Poisons

The effect of the action of a toxic compound on an organism is the occurrence of symptoms of its action. There is practically no symptom that is specific for a single specific compound. Recognised symptoms of the action of a toxic compound (i.e. a syndrome of clinical features, toxidrome) can usually be related to poisoning by a given group of compounds. The most frequently mentioned toxidromes are anticholinergic syndrome (antihistamines, scopolamine, atropine, Jimson weed, tricyclic antidepressants), cholinergic syndrome (organophosphate compounds, derivatives of carbamic acid, mushrooms), hallucination syndrome (ring derivatives of amphetamine, LSD, hallucinogenic mushrooms, Diviner's sage, cocaine, cannabinols), opiate syndrome (opiates, opioids, fentanyl), sedative-hypnotic syndrome (barbiturates, benzodiazepines, ethanol) and stimulant syndrome (amphetamine and its chain derivatives, cocaine). Smellable (odoriferous) and perceptible changes are also useful for directing toxicological analyses. In particular, chemical odour (almonds, garlic, rotten fish or rotten eggs) suggests toxic gases such as cyanide, arsine, phosphine, or hydrogen sulfide, respectively. Discolouration of the skin (redness, hyperaemia) suggests nitrates(III) and nitrates(V); discolouration of the blood and lips (chocolate) is indicative of methemoglobinogenic compounds; and discolouration of urine (red-orange, blue-green, pink-red, dark yellow, dark brown and even black) suggests various medicines or iron compounds. Furthermore, burns (of the skin, oral mucosa, eyes) are indicative of acids and bases, whereas diarrhoea suggests fungi or metals. The size of pupils and their reaction to light are also indicators: dilated pupils indicate amphetamines, atropine and cannabinols, but also opiate withdrawal syndrome; constricted pupils indicate opiates, barbiturates, organophosphates, carbamates and phenothiazines. Bedsores can suggest barbiturate use. Results of analysis of the acid–base balance (acidosis) can indicate methanol and ethylene glycol.

11.2.7 Interpretation of the Results of Toxicological Analysis

Interpretation of the Concentration of Xenobiotic The final stage of the work of the toxicologist-analyst is the interpretation of results. This interpretation must be consistent with the circumstances of the event, the symptoms resulting from the

action of the poison or the autopsy results in cases of fatal poisoning. Most frequently, the analyst interprets the determined concentration of the compound or compounds with reference to a therapeutic concentration or low range of concentrations (if the toxic factor does not have application in medicine) as well as normal (metals) and toxic concentrations and those encountered in cases of fatal poisoning.

Scope of Interpretation Interpretation of results of toxicological analyses for the purposes of judicial decisions encompasses a very broad range of issues. Besides confirming or ruling out the administration of a xenobiotic or exposure to a toxic compound, toxicologists are frequently asked to solve problems such as determining the method (active administration, passive exposure) of introduction of the psychoactive substance (9THC, amphetamine, cocaine) into the organism, determining the source of the compounds (medical treatment, diet, deliberate induction of a state of intoxication), determining the time of the last administration (in cases of frequent administration of psychoactive substances), conducting a retrospective account (e.g. in order to establish the concentration of ethyl alcohol in the body a few hours prior to sampling blood) and commenting on the advisability of carrying out an exhumation for the purpose of confirming or ruling out a possible cause of death from poisoning (e.g. amphetamine derivatives, LSD).

The range of activities of the toxicologist-analyst also encompasses estimating the production capacity of an illegal clandestine laboratory in which controlled substances are produced, as well as determining the yield from cultivation of crops for the production of substances of abuse, such as cannabis.

Profiling The task of the toxicologist-analyst is not only to confirm or rule out poisoning, and identify and quantitatively analyse the main components of non-biological materials secured by the police, but also to characterise samples of drugs by a detailed definition of their physical features and chemical composition (enabling their classification and comparison), and determine the relationship between them. Such a detailed identification of components occurring in trace amounts in samples is known as profiling. Profiling allow explanation of many facts that are significant for judicial bodies, such as the link between two or many samples, the link between a dealer and a drug addict, the distribution network of samples on the local, national or international level (by showing a similarity between samples at the microtrace level), the origin of samples and the method of synthesis. Furthermore, this type of identification is helpful in establishing the geography of the drug trade, tracking the emergence of new sources of substances that are under international control, estimating the size and activity of clandestine laboratories, and distinguishing between substances originating from illegal and legal sources [43].

Thanatochemical Processes When interpreting the results of toxicological analysis of autopsy material, knowledge of putrefactive decomposition processes occurring in corpses (thanatochemical processes) is very important. The direction and intensity of progressive post-mortem changes occurring over time depends on

many factors, among which significant roles are played by the physical condition of the dead person, illness prior to death, injuries, the course of the agony and environmental factors. Moreover, degradative transformations can occur in different ways in different organs of the same corpse. Processes occurring on the surface of organs (aerobic) are faster than those in internal parts (anaerobic). As a result of putrefactive decomposition processes, substances (alcohols such as ethyl, methyl and higher; cyanides) can be produced in corpses that are significant from a toxicological point of view. These substances are of endogenous origin, and thus were not introduced into the organism before death. To date, no specific explanation has been found for the production of cyanide ions at high concentrations in some post-mortem blood samples, comparable with values occurring in cases of poisonings. On the other hand, as a result of degradation of biological material, many toxic compounds that had been taken while the person was still alive (i.e. compounds of exogenous origin) undergo decomposition. These are esters of higher alcohols, numerous volatile organic compounds and many drugs, especially with a quaternary ammonium base structure. As a result of degradation of amino acids, which are natural components of biological material, alkalisation of the environment occurs, which at the stage of far-reaching putrefactive decomposition of biological material makes it impossible to draw conclusions about poisoning by acids, especially hydrochloric acid [44].

Endogenous Compounds Interpretation of the results of toxicological analysis of compounds that are natural components of the organism, but also poisons, can be a big challenge for forensic toxicologists.

The physiological level of ethyl alcohol is of the order of 0.01 ‰, whereas in persons suffering from diabetes or in hunger states, it can be somewhat higher, but never exceeds the statutory threshold of a state indicating consumption of alcohol (i.e. 0.2 ‰). However, as a result of production of endogenous alcohol in corpses, interpretation of its origin is sometimes very difficult. In order to establish whether we are dealing with alcohol produced post mortem (in vitro) or consumption of alcoholic drinks before death (in vivo), two types of post-mortem material should be subjected to analysis: blood (in this material, production of endogenous ethyl alcohol occurs very quickly after death) and vitreous humour (whose decomposition is slower), or blood and urine.

Methanol, a poison acting through its metabolites, is a component of all alcoholic drinks produced by the descendant companies of the Polish Spirits Monopoly, and occurs naturally in living organisms. The origin of endogenous methanol has not yet been unambiguously explained. Its concentrations range from 0.1 to 3.4 mg/kg (i.e. several orders of magnitude lower than concentrations of methanol occurring in cases of poisonings).

The carbon monoxide derivative of haemoglobin (carboxyhaemoglobin, HbCO) also occurs in small amounts (2–7 %) as a natural component of the living organism, being a product of the decomposition of haemoglobin. HbCO can also be produced as a result of inhaling carbon monoxide from the environment (in the case of smoking cigarettes, HbCO reaches a concentration of the order of 11–13 %),

or through post-mortem decomposition processes (leading to values of the order of 5–7 %). Long-term exposure to carbon monoxide causes severe poisoning by carbon monoxide, but the concentration of HbCO may be very low (not exceeding 2 %).

Gamma-hydroxybutyric acid (GHB) is also a natural component of living organisms. In the past it was used in medicine, whereas currently this compound is increasingly abused for recreational purposes, and as a date-rape drug. It occurs naturally in the organism in very diverse concentrations, which change during storage of biosamples collected when the subject was alive and from post-mortem examinations. Furthermore, it undergoes rapid elimination, even when administered at very high doses (detectable up to 8 h in blood and up to 12 h in urine). For these reasons, GHB causes very great interpretation difficulties in relation to its in vitro or in vivo origin [45].

Competency of the Analyst The forensic toxicologist-analyst interprets the results of analysis within his or her competency, in relation to general toxicological knowledge and the general population. A medical doctor specialising in clinical toxicology pronounces on the defined behaviour of a specific person under the influence of a given drug. Solving problems relating to criminal responsibility (type of punishment, offence, indictment, prosecution, ban) lies clearly within the remit of the adjudicating body (the court), in other words, issues such as substantive assessment of the validity of conclusions in the case of conflicting opinions of two experts.

11.3 Contemporary Toxicological Analysis

Fundamentals of Toxicological Analysis Toxicological analysis is the cornerstone of clinical and forensic toxicology. Currently, a forensic medical doctor will not issue an opinion on the cause of death as a result of poisoning without the results of toxicological analysis of post-mortem material. Without analysis of body fluids taken from a living person suspected of having been poisoned, it is impossible to issue a categorical opinion. In clinical toxicology, the results of analysis most often serve to diagnose poisoning and monitor the success of treatment, but in cases of hospitalisation of victims and perpetrators of various criminal activities, the results of analysis are used for the needs of the administration of justice. The evidential value of the results of toxicological analysis depends on the method applied for determinations and on the type of material collected for study (as a result of legal, scientific and analytical requirements, etc.).

The basis of modern toxicological analysis is a two-stage examination of biological and non-biological material. In the first stage, screening methods are applied, and in the second stage, confirmatory methods. The analyst uses screening methods to obtain preliminary results (i.e. non-categorical results; negative and positive), after which the positive results must be verified by confirmatory methods.

Screening methods are aimed at analysing as broad a spectrum of various chemical compounds as possible. Confirmatory methods are more specific and are characterised by a lower limit of detection (LOD) and limit of quantification (LOQ) than screening methods.

Validation of Methods For any analytical process to be applied for forensic toxicological purposes, it must be subject to control from the moment of collecting the sample to obtaining the result and then documenting this result. Standardisation of the method (i.e. validation) ensures such control. Thus, validation parameters must be determined for each new method (screening, confirmatory, qualitative and quantitative) in accordance with international requirements. According to the Scientific Working Group for Forensic Toxicology (SWGTOX), parameters such as the following must be determined for all methods used in the analysis of biosamples: precision, dilution integrity, interference studies, LOD, carryover and stability. For quantitative methods, the following are required: bias, calibration model and LOQ [46]. The following are considered to be additional parameters that should also be determined: recovery, reproducibility and sensitivity of the method to small changes (ruggedness or robustness). For methods using LC-MS, the matrix effect should always be studied, in other words, ionisation suppression or enhancement, especially in an electrospray ionisation (ESI) chamber [47]. Applying deuterated derivatives of analytes as internal standards, checking the correctness of methods by analysis of reference materials and participation in interlaboratory comparisons facilitates continuous control of result uncertainty .

Validation of a method is not research work, but an integral part of the process of quality assurance of results of examinations in every modern analytical laboratory applying principles of good laboratory practice, and constitutes a condition that is necessary both for gaining accreditation and for recognition (in the international arena) of results of examinations conducted in the laboratory. Determination of validation parameters undoubtedly prolongs the period of development of a method, but is essential to confirm its usefulness for achieving the intended analytical goal. This process does not conflict with Albert Einstein's statement that "everything should be made as simple as possible, but not simpler". On this basis, simplification of the method by omitting the process of validation is not permissible.

Material Selection of appropriate material for examination is mainly determined by the time that has elapsed from administration of the toxic substance to collection of the material, as well as the site of conducting examinations (a clinical or forensic laboratory, or the site of the incident, for example, during a roadside check of a driver). Various compounds occur in various materials – parent, and active and inactive metabolites. Active metabolites influence life processes, and the presence of inactive metabolites in the organism could attest to consumption of a substance a long time ago. Currently available analytical techniques are applied to detect, identify and determine chemical substances in classical biological material (i.e. blood, urine and sections of internal organs) as well as in so-called alternative materials (i.e. hair, saliva and sweat). In recent years, numerous studies aimed at

showing the correlation between concentrations of various compounds in saliva and blood have been conducted [48]. Assuming that a given poison is excreted via sweat, a pillowcase can also be a useful, indirect material for toxicological tests. Many analysts undertake analysis of fresh or old blood stains revealed on various materials. It is worth mentioning that modern analytical methods allow conclusions to be drawn about the cause of death as a result of poisoning on the basis of analysis of fly larvae developing on human remains [49, 50]. This material also serves for estimation of the time of death, both in hot [51] and cold climates [52].

Sometimes the amount of material delivered for examination is very limited for completely unjustified reasons. When developing methods, analysts take this fact into account. Increasingly frequently, blood samples of only 1 mL are collected for screening analyses, whereas as little as 0.1 mL blood is sampled for targeted or confirmatory analyses.

Directions of Analysis Analytical procedures depends on the type of problem set. Unknown circumstances of an event or an unknown toxic factor require the application of systematic toxicological analysis (STA), so that the analytical procedure encompasses as many toxic substances as possible. In cases where the administered toxic compound is known, first of all a course of analysis targeted at this compound is conducted, and a positive result must be confirmed by another independent method. When working on a case in which only the symptoms of the action of an unknown toxic factor are given, the ability to use complementary techniques as well as knowledge of the fields of medicine, pharmacology and pharmacokinetics are of particular importance [53].

11.3.1 Screening Methods

Immunochemical Methods Commercial tests that make use of immunochemical reaction (ICh), enzyme immunoassay (EIA), radioimmunoassay (RIA), fluorescence polarisation immunoassay (FPIA) and the kinetic interaction of microparticles in solution (KIMS) are most frequently applied as screening methods. They are designed to detect compounds from defined, but not numerous, groups (e.g. opiates, cannabinols, derivatives of amphetamine, tricyclic antidepressants and benzodiazepines), and, more rarely, single compounds (e.g. digoxin). These tests enable very quick analysis of a specific body fluid (urine, serum or saliva). The obtained result relates to the whole group of compounds, and is defined as group-positive or group-negative. Depending on the method of detection (visual or electronic reading using an analyser), the obtained result can be quantitative, semi-quantitative or only qualitative.

ICh tests have many advantages: they are very sensitive, rapid, do not require pre-treatment of the biological material (which they are designed to analyse), and use small quantities (0.01–0.1 mL) of body fluids. Their principal drawback is low specificity.

Principles of Immunochemical Tests All ICh tests use an antibody or other binding protein, an antigen and a label. The basic principle underlying these tests is competition between the unlabelled antigen (the drug from the biosample) and the labelled antigen for a binding site in antibodies. The type of label (radioactive, chemical and fluorescent labels are used) with which the antibody or antigen is labelled determines the detection technique. In RIA tests, labels are nuclides (^{3}H,^{14}C,^{125}I,^{131}I) introduced into the antigen, antibody or enzyme. Tests using measurement of radioactivity are very sensitive. When using a fluorescent label or luminophore we measure, respectively, a change in fluorescence in polarised light (FPIA) or chemiluminescence. Various types of enzymes can also be labels. The principal components are the components of the sample, antigen labelled with a specific enzyme, a specific antibody for the antigen and a substrate that causes a measurable change in optical signal when it takes part in a reaction catalysed by the enzyme. In tests involving measurement of absorbance, the co-presence in the sample of compounds characterised by a high molar absorbance coefficient influences the result, causing a false positive result to be obtained. Chemical compounds or dyes that effect a change in colour of a solution or indicator zone can also be used as labels. The simplest tests make use of test strips (e.g. the American Frontline test) that are immersed in a urine test sample. On the paper strip, the components of the sample are subject to chromatographic processes and reach the zone where antibodies are deposited. The drug from the urine binds with antibodies. The surplus of free antibodies reaches the second zone, in which antigens are immobilised. The antigens capture all unbound antibodies. Only particles of antibody bound to the drug from the sample undergo further chromatographic processes. These conjugates reach the label zone, which often contains colloidal gold as a label. A change in colour of this zone occurs, whereby a red band indicates a positive result. In other strip tests (e.g. Hydrex), in spite of the same label, the appearance of a coloured band indicates a negative result.

Chromatographic Methods Chromatographic methods such as thin layer chromatography (TLC), GC and HPLC, with various types of detection, are more universal and specific, especially when they are coupled with MS. These methods, with the inclusion of MS, are defined as open (i.e. they allow inclusion of successive new compounds into a previously developed analytical procedure). Screening methods using both GC and HPLC for separation and, for detection, tandem mass spectrometry (MS/MS) with selected ion monitoring (SIM), selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) encompass a limited number of compounds. This is often a result of the limitations of the equipment, which restrict the number of analytical signals (ions or reactions) that can be simultaneous recorded at an appropriately high sensitivity in one process. That is why in recent years screening methods targeted towards a strictly defined group of compounds have been developed in place of so-called general screening methods. Examples include a method for detecting substances affecting the psychomotor performance of drivers, and a method for detecting substances use to facilitate rape or robbery. This approach ensures that the sensitivity of the screening method is

satisfactory, although all screening methods are less sensitive than confirmatory methods or methods targeted towards a specific compound.

Note that with the need to analyse an ever-increasing number of analytes at increasingly small concentrations (trace analysis), various methodological pitfalls arise [53].

Spectrometric Methods Screening methods are not just restricted to organic compounds. In addition to flame (F-AAS) and flameless atomic absorption spectrometry (including cold mercury vapour atomic absorption spectrometry, CV-AAS), which are established methods for studying metal content in biological material, other techniques such as inductively coupled plasma optical emission spectrometry (ICP-OES) or inductively coupled plasma mass spectrometry (ICP-MS) are used increasingly frequently. The latter two techniques enable analysis of about 70 elements in one analytical process, the specific number being dependent on the number of standard substances that the analyst has at their disposal. The method of choice for determination of Hg, As and Se is AAS with hydride generation (HG-AAS), whereas AAS with electrothermal atomisation (ET-AAS) enables determination of trace amounts of heavy metals not only within the normal range, but also at levels encountered in chronic poisoning and, in some cases (e.g. Tl, Pb or Se) in acute poisoning. In order to rule out or confirm a poisoning, especially a chronic poisoning, by toxic metals, semi-metals and non-metals, knowledge of normal levels of elements occurring in trace amounts in particular types of biological material is essential.

Systematic Toxicological Analysis The number of toxic compounds that must be taken into account in cases where the circumstances of an incident are completely unknown (e.g. a corpse found in a forest, an unconscious person found in a park) is continuously growing. It is not possible to encompass all the various chemical compounds that are significant from a toxicological point of view in one analytical process.

Toxic substances can be classified in various ways (e.g. alphabetically) or in terms of pharmacological activity (tricyclic antidepressants, anticonvulsants, antihypertensives) or chemical structure (derivatives of benzodiazepine, barbituric acid, phenothiazine). For the purposes of STA, the best way seems to be to divide compounds into groups depending on the type of technique used to extract them from various biological materials. This gives six basic groups [53]. These groups are listed below in the (chronological) order that should be maintained when performing toxicological analysis:

- Gases and volatile compounds, which can be isolated by diffusion into the headspace
- Toxic anions such as nitrates(III), nitrates(V), phosphides and oxalates, isolated by dialysis
- Sparingly volatile organic compounds, for which the most suitable method is pH-dependent extraction with organic solvents (liquid–liquid extraction, LLE) or the solid phase extraction (SPE) screening technique

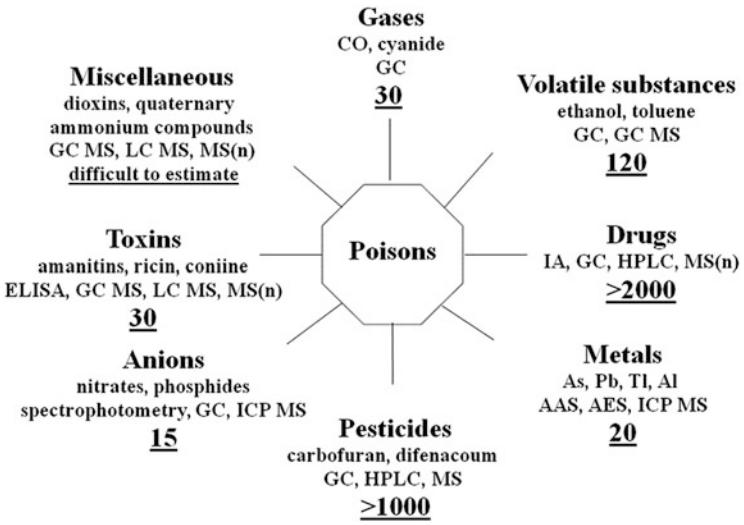

Fig. 11.10 Classification of poisons for analytical purposes. The most commonly used methods for their determination and estimated numbers of toxicologically relevant compounds (*underlined*) are indicated

- Pesticides, although many require a defined extraction procedure because a general procedure is not effective
- Metals and non-metals, which require application of various mineralisation techniques (wet, ashing, microwave-assisted)
- Toxins and a large group of compounds (e.g. quaternary ammonium bases and dioxins) that require special isolation techniques, using ion pairs or ion exchange resins, formation of derivatives, continuous extraction, precipitation and concentration

Detecting or ruling out the presence of a broad spectrum of compounds from each of these groups in biological material (Fig. 11.10) requires the application of increasingly sensitive instrumental techniques.

11.3.2 Confirmatory Methods

Modern instrumental techniques, particularly the coupling of GC-MS, LC-MS or LC-MS/MS with various types of ionisation (electron, EI; chemical, CI; electrospray, ESI; photoionisation, APPI) leading to the formation of positive and negative ions, as well as various modes of monitoring these ions (total ion current, TIC; selected ion monitoring, SIM; parent ion scanning, PIS; daughter ion scanning, DIS; selected reaction monitoring, SRM), enable – in one analytical process – separation of a mixture of compounds, identification of individual components and

their determination at concentrations of the order of picograms in tens of microlitres (of blood, serum, urine) or milligrams of material (hair). Methods using these techniques require preliminary, multistage (hydrolysis, extraction, derivatisation) preparation of biosamples, application of appropriate standard and reference materials and determination of validation parameters, which ensure control of the whole analytical process. Such methods are thus very time-consuming and costly. Constantly improved design solutions for mass analysers (quadrupole, Q; ion trap, IT; triple quadrupole, Q_3; time of flight, TOF; Fourier-transform ion cyclotron resonance, FT-ICR) enable lowering of the LOD to very low values.

11.3.3 Identification Systems

Each laboratory should have its own analytical procedures. These procedures can be based on commercial or published systems, but their reproducibility and sensitivity (robustness) in new laboratory conditions must be checked. To date, most systems have been developed for screening analysis of organic compounds. Numerous systems of identification have been developed for one of the oldest chromatographic methods (TLC), each encompassing over 1000 different compounds, including medicines and their metabolites [54]. For the GC method with classic detection, two systems are best known. One method is used in screening analysis for the presence of sparingly volatile organic compounds and is based on two types of detectors (flame-ionisation detector, FID, and nitrogen phosphorus detector, NPD) and retention indices of 4500 compounds [55]. The second method is used for the identification of volatile organic compounds of the solvent type [56]. Computerised systems of identification of drugs can be purchased with liquid chromatographs equipped with diode array detectors (HPLC-DAD). Using these systems, compounds divided into acidic and basic (encompassing neutral compounds in both groups) are identified on the basis of the relative retention time and spectrophotometric spectrum. Another significant HPLC-DAD system, compatible with high performance chromatography systems from various companies, encompasses about 2682 compounds, taking into account, in addition to the above mentioned identification elements, the molecular structure by comparing 1600 chromophores or combinations thereof [57, 58].

Chromatographic techniques coupled with mass spectrometry (GC-MS, GC-MS/MS, LC-MS and LC-MS/MS) have also been applied in many combinations. A library of reference mass spectra is an integral part of each type of GC-MS apparatus, enabling typing of spectra that are potentially similar to the identified compound. For the GC-MS-EI technique, a combination of two previously separate libraries, Wiley and NIST 2008 (W8/N08), should be mentioned because of its extensiveness. Library W8/N08 contains 562,000 EI spectra, 5308 spectra of parent ions (precursors) subjected to fragmentation by the MS/MS technique, over 2 million names of chemical compounds and their synonyms, 35,000 structural formulae and 43,000 GC retention indices. The Automated Mass Spectrometry

Deconvolution and Identification System (AMDIS) is also integrated into this library. The Pfleger/Maurer/Weber library is most useful for the needs of toxicological analysis, containing 7800 spectra of drugs, pesticides, their metabolites, derivatisation products (especially methyl and silyl derivatives) and artefacts (compounds that might be produced during analysis, for example, under the influence of high temperature in the injection chamber of the chromatograph). This library also contains other data on compounds, such as Kovats retention indices, structural or empirical formulae, molar mass, the Chemical Abstract Service Registry Number, the name of the pharmacological group into which the compound is classified, the type of biosample and a description of the method of preparation of the sample [47]. In spite of this, not all parent compounds and their metabolites from this library can be encompassed by one analytical procedure.

When developing an analytical procedure using the LC-MS technique, it should be remembered that mass spectra after ionisation of a given compound in the ESI chamber or in APCI conditions obtained after single fragmentation contain, in contrast to EI-type spectra in GC, very few fragments. The pseudomolecular ion obtained in APCI mode is also characterised by low identification value. The identification value of spectra increases after the application of multiple fragmentation and collection of spectra of daughter ions. An important problem for ESI is the possibility of reduced ionisation of the analyte by co-present compounds in the sample, which is called suppression of ions and can lead to overlooking a strongly toxic compound whose concentration in the sample is low. Bearing the above in mind, a library of daughter mass spectra (MS/MS) of the ESI type with fragmentation within the source by the method of collision-induced dissociation is very useful [59]. The library contains spectra of over 800 (pharmaceutical) drugs at three (low, medium and high) collision energies. Furthermore, the same authors have created a library of mass spectra for the LC-MS-Q method, whereas Schreiber [60] has established a library of ESI and APCI-type spectra for identification of pesticides and explosive compounds. These libraries have been commercialised. For LC-MS or LC-MS/MS methods, most libraries have so far been created in a "homemade way" in individual laboratories. These libraries work well for particular instruments or the same types of instruments.

11.3.4 Analytical Strategies

Often in identification systems, one pre-treatment procedure is applied to samples, but various end determination techniques (GC, HPLC and, increasingly rarely, TLC). Because of the high sensitivity and broad range of linearity (often encompassing three orders of magnitude) of coupled techniques (GC-MS or LC-MS), some analysts apply one method of sample preparation (extraction and derivatisation) to all analytes, in spite of the low efficiency of the process of isolation of acid compounds subjected to extraction from an alkaline medium. The most extensive method to date, enabling detection and identification of over

2000 compounds (medicines and their metabolites from 20 pharmacological groups) in one extract (pH 8–9) from urine, was developed by Maurer et al. using the GC-MS-EI technique [61]. With the help of modern liquid chromatographs, it is possible to record mass spectra under planned changing measurement conditions (e.g. at two fragmentor voltages) in the course of one process. The basis of other analytical procedures is the application of complementary methods using different types of detection (MS/MS, electron capture and FID) and ionisation (EI, APCI and ESI). Numerous analysts are advocates of pH-dependent extraction and combining extracts before instrumental analysis, or their separate analysis. In many laboratories, methods designed for studying a defined type of biological material (blood, urine, saliva or hair) are being developed with maximum a posteriori probability (MAP) [62], as well as for studying specific pharmacological groups (e.g. benzodiazepines [63, 64], antidepressants [65], beta-blockers [66]). More and more developed methods relate to a specific problem and biological material. For example, the LC-MS-APCI method serves to detect and determine substances facilitating commission of crime (rape, looting, robbery) in blood [41] and urine [67]. The LC-MS-ESI method determines substances acting similarly to alcohol in drivers' blood [68], such as phenylalkylamines of plant origin in plasma [69]; phenylalkylamines considered designer drugs classified into group 2C and, more precisely, containing two dimethoxy groups attached to the benzene ring in positions 2 and 5, in plasma [70]; and α- and β-amanitines (Death Cap toxins) in urine [71]. Coupled techniques enable the development of universal methods that allow screening analysis, identification and quantitative analysis to be conducted in one process. In the case of the LC-MS, the successive stages of the procedure for one extract from a biosample are screening analysis, in which suspected compounds are typed, and then identification of these compounds using the total ion current mode (TIC, SCAN). For quantitative analysis of a compound identified in this way, it is sufficient to monitor one ion per compound. Among the most extensive screening methods developed to date using the LC-MS technique are the LC-MS/MS-QTrap method, encompassing 301 compounds in blood and urine [59], and the LC-MS/MS-ESI method [72], encompassing 238 drugs in blood. Alder's team [73] developed and compared two methods, LC-MS/MS and GC-MS-EI, for the identification of 500 of the most frequently applied pesticides. Pang et al. [74], applying gel chromatography to preliminary separation, conducted validation for 660 pesticides and then, using quantitative analysis by GC-MS and LC-MS/MS, encompassed 437 active components of plant protection agents, dividing them into four groups.

11.3.5 Summary

As a result of the increasing number of samples that are subjected to toxicological analysis, efforts are being made to automate the analytical process. Software is being developed to aid the measurement process by automatic tuning, collection, editing and archiving of data; creation of reports; searching through libraries; and

quantitative analysis. Extensive literature and internet publications on the subject are available. Continuously developed analytical procedures are verified by internal and external systems of quality control for the results of analyses. All this enables forensic analyst-toxicologists to screen about 3000 compounds in the everyday work of a typical well-equipped laboratory, in cases where there is a lack of information about a particular event. The remaining compounds of significant toxicological importance, whose number is estimated at about 100,000, require application of a specific analytical procedure targeted towards a given compound.

Although analysts know the analytical canons and methods, they are conscious of methodological traps and apply generally accepted analytical procedures for seeking and identifying poisons. Moreover, they detect poisons with the help of biomarkers of exposure, effects and sensitivity in biological material, enabling confirmation of poisoning in the period of occurrence of symptoms (in blood, saliva and urine) or in the period after symptoms of poisoning have subsided (in urine, hair and sweat). General knowledge, incisiveness, an ability to discern logical associations between facts and, increasingly rarely, chance play important roles in determining whether or not analysts are successful in their investigations.

Currently, toxicological analysis of biological material increasingly frequently boils down to the need to detect, identify and determine trace amounts of toxic compounds. Criteria of identification of compounds vary, depending on current knowledge and new requirements, which in turn depend on the field and the problem to be solved. It is obvious that the most stringent requirements are set in those fields in which results lead to legal sanctions. In these fields in particular, the analyst-toxicologist should make use of the best knowledge and fulfil all formal conditions. After their fulfilment, the result of identification should be described in terms such as "nothing stands in the way of stating that it is this compound" and, in the case of a quantitative result, "the compound at the determined concentration could have caused a health disorder or threat to life, but it is not an unequivocal condition". The analyst, acting within his/her strictly limited remit, works in partnership with a forensic medical doctor in issuing opinions for judicial bodies. It is currently difficult to imagine a ruling on the cause of death as a result of poisoning without, at the very least, a toxicological analysis of post-mortem material. In such cases, the consistency of factual findings, the picture of the autopsy and the results of toxicological analysis authorise the forensic medical doctor to issue an opinion about the cause of death. The toxicologist-analyst can assess the number of active single doses of the substance of abuse or psychoactive substance, estimate the yield of crops cultivated for the purpose of producing substances of abuse (cannabis) or the production capacity of an illegal laboratory producing a controlled substance (amphetamine), but a substantive assessment of the results of these investigations in relation to criminal liability clearly lies within the remit of the adjudicating body.

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Part III
Special Aspects of Trace Analysis

Chapter 12
Inorganic and Bioinorganic Speciation Analysis: Problems and Prospects

Lena Ruzik, Katarzyna Pawlak, and Maciej Jarosz

Abbreviations

AAS	Atomic absorption spectrometry
AdTSV	Adsorptive transfer stripping voltammetry
AFS	Atomic fluorescence spectrometry
AsB	arsenobetaine
ASE	Accelerated solvent extraction
ASV	Adsorptive stripping voltammetry
CE	Capillary electrophoresis
CEC	Micellar electrokinetic chromatography
CRM	Certified reference material
CZE	Capillary zone electrophoresis
DMA	Dimethylarsinic acid
EI	Electron ionization
ESI	Electrospray ionization
FPD	Flame photometric detector
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HPLC ESI	High-performance liquid chromatography/electrospray ionization
MS	mass spectrometry
IC	Ion chromatography
ICP MS	Inductively coupled plasma mass spectrometry
IPLC	Ion-pair liquid chromatography
IR	Infrared
LLE	Liquid–liquid extraction
MEKC	Micellar electrokinetic chromatography

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MIP OES	Microwave-induced plasma optical emission spectrometry
MMA	Monomethylarsonic acid
MS	Mass spectrometry
NPLC	Normal phase liquid chromatography
OES	Optical emission spectrometry
PLE	Pressurized liquid extraction
PSA	Potentiometric stripping analysis
RPLC	Reversed phase liquid chromatography
SDME	Single drop micro-extraction
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SLE	Solid–liquid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
Tris	Tris(hydroxymethyl)aminomethane

12.1 Speciation and Speciation Analysis

Speciation refers to the various physical and chemical forms in which an element can exist in a real-world material. The forms of an element are its manifestations differing in isotopic composition, electronic structure or oxidation number, or the structure of the molecule or complex [1]. Speciation analysis is the procedure of identifying individual forms of a particular element and their quantification. The above definition concisely outlines the purpose of an analyst's activities. However, the quality of identification of compounds is often dependent on the available equipment, the general state of knowledge about the biochemistry of the investigated element, and the experience of the analyst. Correct identification is a challenging task for many reasons, one of them being that it is necessary to determine the properties of a compound of an element present at the trace level, and inadequate sensitivity is the most common drawback of molecule-specific techniques. Another problem is that analyses often seek to determine the identity of compounds that have not yet been described in the literature. If this is the case, the practical analytical procedure must ultimately isolate the chemical form being searched for, and obtain it in a purity that is typical of the standard substance. The process usually involves several stages:

1. Representative sampling of a material for analysis and sample stabilization
2. Specific extraction (leaching) of compounds of the investigated element from the analyzed material
3. Separation of extracted compounds by liquid chromatography at a semipreparative or preparative scale, followed by inductively coupled plasma mass spectrometry (ICP MS) or inductively coupled plasma optical emission spectrometry detection for examining the presence of the element in individual fractions

4. Concentration of isolated fractions containing the forms of the element, followed by another chromatographic purification (one- or multidimensional)
5. Identification of the forms thus obtained using complementary molecule-specific techniques (nuclear magnetic resonance; infrared [IR]; matrix-assisted laser desorption/ionization; electrospray ionization [ESI]/atmospheric pressure chemical ionization mass spectrometry [MS])
6. Assessment of purity of isolated substances by high-performance liquid chromatography (HPLC) ICP MS

If appropriate standards are available, the above-described procedure for determining the structure of the forms is not necessary. In that case, the following stages are typically performed:

1. Taking a representative sample of material for analysis and sample stabilization
2. Specific extraction (leaching) of compounds of the investigated element from the analyzed material
3. Chromatographic separation (by HPLC, most commonly with ICP MS detection) of the forms of the investigated element that are present in the extract of analyzed material; at this stage, it is vital to select a method providing appropriate resolution
4. Identification of the separated forms by the addition of an internal standard and their preliminary semiquantification
5. Verification of correct identification and semiquantification by an independent chromatographic method
6. Quantification of the isolated forms by means of two chromatographic methods, taking into consideration the matrix effect

The likelihood of error in such complex procedures is considerable. It is significantly higher when identification is preceded by activities involved in sampling and preparation of material for analysis. The second stage in solid material testing usually comprises multistage leaching with solutions of increasing elution power (i.e., fractionation). During this stage and during storage, the material can undergo spontaneous changes, often induced by impurities introduced by the analyst. In consequence, the identified compound can be a product of biochemical or physical transformations taking place during the analytical procedure. In these circumstances, the goal of speciation studies can be defined as the "conceptual extrapolation" of the results to the probable initial state, typically performed in cooperation with biologists, physicians, toxicologists, and others. The above problem is disregarded in certain cases, for example, during fractional soil analysis when the aim is to determine the degree of absorption capacity of a particular element by plants rather than to identify the element's forms. The risk of errors also stems from the fact that the scope of speciation analysis encompasses a variety of materials (items of environmental origin, food, body fluids, etc.) and the broad range of compounds found in them. Because of complicated procedures, which are developed on a case-by-case basis, errors tend to originate in the negligent performance of basic laboratory activities rather than in the limited state of knowledge or lack of access to appropriate molecule-specific techniques.

Studies of speciation to date have investigated combinations of only about 20 elements (chiefly Al, As, Cd, Co, Cu, Pb, Hg, Pt, Se) and four groups of their compounds, including halogens, volatile compounds, and complexes with peptides, proteins, or sugars [2]. One of the most common metalloids studied is arsenic, because of its toxic characteristics and widespread presence in water, plants, and seafood. The element occurs in environmental materials in a variety of physical and chemical forms, ranging from inorganic salts or toxic acids [As(III) or As(V)] to harmless arsenobetaine (AsB) or complexes with peptides. More than 50 different arsenic compounds synthesized by plants have been detected over the past 30 years. Therefore, it is not surprising that speciation analysis of the element, aimed at identification and determination of its forms, gives rise to a multitude of problems [3–8].

Arsenic in the environment has two basic sources: (1) natural (e.g., Poland's Kłodzko Valley abounds in arsenic-rich rocks, which results in a considerable content of arsenic in the region's drinking water [9, 10]); or (2) anthropogenic, caused by entry of arsenic into the soil or waters, as a result of human activity, in the form of herbicides or pesticides [10, 11], fungicides, products and waste from the electronic industry (e.g., semiconductor materials), and wastes from the tanning industry [11, 12]. Variation in the toxicity of arsenic compounds and their diverse capacity to accumulate in living organisms are major stimuli triggering the development of speciation analysis of this element, especially considering that the organisms are a basis of human diet. These considerations also call for the unification/standardization of analytical procedures proposed for the determination of different forms of arsenic in samples of geological [11–14], environmental [13–16], biological [15–18], and clinical origin [17–21] and in food products [9, 16, 20–24].

The degree of complexity of a material considered from the analytical (speciation-oriented) viewpoint stems not only from the intricate nature of its matrix but also from potential biochemical processes leading to spontaneous transformations of toxic compounds, which are often determined by the origin of samples. In tests of geological materials (including groundwater), for example, the presence of inorganic compounds of As(III) or As(V) is to be expected [25, 26]. Unconfined groundwater can contain the more toxic methyl derivatives of As(V) acid [27] and less toxic derivatives of As(III) acid [28], arising in the process of biomethylation of arsenic. In the world of plants, the number of forms of the element increases because plants synthesize arsenosugars, arsenolipids, AsB, and arsenomethionine. This is an effect of the similarity between arsenic and phosphorus, leading to the substitution of phosphorus atoms with arsenic atoms. Four arsenosugars most commonly occurring in marine biota are formed via successive alkylation and adenosylation processes: arsenosugar A – 3-[5'-deoxy-5'-(dimethylarsinoyl)-b-ribofuranosyloxy]-2-hydroxypropanesulfonic acid, arsenosugar B – 3-[5'-deoxy-5'-(dimethylarsinoyl)-b-ribofuranosyloxy]-2-hydroxypropylene glycol, arsenosugar C – 3-[5'-deoxy-5'-(dimethylarsinoyl)-b-ribofuranosyloxy]-2-hydroxypropyl hydrogen sulfate, arsenosugar D – 3-[5'-deoxy-5'-(dimethylarsinoyl)-b-ribofuranosyloxy]-2-hydroxypropyl 2,3-hydroxypropyl phosphate. Another examples of the most commonly identified or assayed arsenic compounds are listed in Table 12.1.

Arsenic is present mainly in ionic forms, which are usually separated by high-performance ion-exchange chromatography coupled to an atom- or isotope-specific

Table 12.1 The most common forms of arsenic [29]

	Abbreviation	Chemical structure	Toxicity	References
Arsenic	As		Carcinogenic	[25, 26]
Inorganic compounds				
Arsenite ion (III)	As(III)	$AsO(OH)_2^-$		
Arsenate ion (V)	As(V)	$AsO_2(OH)_2^-$		
Organic compounds				
Monomethylarsonic acid(V)	MMA(V)	$CH_3AsO(OH)_2$	Carcinogenic	[27]
Dimethylarsinic acid (V)	DMA(V)	$(CH_3)_2AsO(OH)$	Carcinogenic	[30]
Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COOH$	Nontoxic	[31]
Arsenocholine	AsC	$(CH_3)_3As^+(CH_2)_2COOH$	Nontoxic	[32]
Trimethylarsine oxide (V)	TMAsO	$(CH_3)_3AsO$	No data	
Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$	No data	
Trimethylarsine		$(CH)_3As$	No data	
Arsenosugars		Different structures	No data	
Arsenolipids			No data	

detector (e.g., OES or MS with excitation/ionization in ICP). This stage, however, is preceded by a number of earlier stages that can give rise to serious errors.

12.2 Sampling and Storage of Samples

The first stage of the analytical procedure involves representative sampling of material. This stage is of key importance for the quality of results and must be performed in a manner ensuring minimum loss of the assayed element and maximum protection from contamination, including impurities that could upset the delicate equilibria existing between active forms.

A major role in speciation analysis is played by appropriate selection of laboratory ware (material, color) used for sampling, storing samples, and maintaining their purity. The problem of contamination or loss of assayed elements at this stage depends for the most part on the susceptibility of analyzed compounds to ultraviolet (UV) radiation and oxidation through contact with air. Other factors that need to be considered include the possibility of evaporation of the element, its transformation into a different form, and absorption/adsorption on the vessel wall. All vessels should be thoroughly washed with deionized or distilled water or, if the nature of the sample allows, with 10 % nitric acid.

Gaseous samples are typically taken by means of isolation techniques (using gas pipettes, ampoules, plastic bags, or canisters with walls lined with an inactive polymer) or aspiration techniques (absorption on solid sorbents or in appropriate

solutions, freezing in cryo-traps). Such operations can potentially lead to analyte loss as a result of photolysis, hydrolysis, oxidation, adsorption on vessel walls, absorption, or other processes. Losses can be minimized by controlling appropriate parameters such as temperature, light, humidity, or oxygen content [33]. The safest method of storing gas samples designated for speciation analysis is in cold traps (liquid nitrogen) for a maximum of several days [34].

Liquid samples should be taken and stored in containers made of materials whose components do not migrate into the analyzed solution. For assays of metal forms, polycarbonate vessels are recommended; however, when mercury compounds are analyzed, glass is a more appropriate material [35].

In analyses of natural waters, the matrix is a major potential source of errors. As a result of constant contact with soil, rocks, and the atmosphere, water contains almost all chemical elements. Furthermore, water is not a homogeneous system (this also applies to water treated for drinking purposes) because it contains hydrated ions, particles, colloids, and even suspensions of small solid particulates. Other components include inorganic complexing substances, such as carbonate, chloride, sulfate(VI), and phosphate ions, and organic compounds such as amino acids, sugars, urea, humic acids, and fulvic acids. For deep underground waters, one should also expect a high content of carbon(II) oxide and a low content of oxygen. It is necessary to maintain water at a natural low temperature range and under elevated pressure. Modification of physicochemical conditions during sample storage can result in a change of form of the investigated element.

The storage of liquid biological materials also carries the risk of introducing major errors into the analysis, because such materials contain a range of biochemically active substances (enzymes, bacteria, and others) that can trigger physicochemical transformation of the compounds under investigation. These reactions are prevented by UV irradiation, shock freezing, or lyophilization (the process of removing water and certain volatile organic substances from the material with the omission of the liquid state). Samples can be stored over a short period at a temperature of +4 °C, in the dark.

One of the most challenging stages of speciation analysis is sampling of solid material in a manner that preserves its representative nature [36, 37]. Correctly sampled biological material frozen in liquid nitrogen should be stored in a neutral gas atmosphere, at a temperature of −20 °C, in polyethylene or polycarbonate bottles. The stage of preparing material for analysis should be as short as possible. There are also other factors (temperature, light, pH) that can induce transformation of the forms of investigated elements or reactions between analytes, components of the matrix, and packaging.

Summing up, samples must be stored in a manner that does not affect their representativeness. The rule applies not only to freshly collected samples but also to extracts obtained at every stage of concentration or purification. It is also important to note that there are no universal methods. Therefore, methods should be optimally adjusted to the compounds being assayed. This demanding task is addressed next using the example of arsenic compounds.

Arsenic is mainly present in different water types (drinking water, surface and groundwater, rainwater, and wastewater), in soil, dust, plants, and fluids of biological origin (blood, milk, urine) [38]. Samples should be stored in vessels of amber glass, polyethylene, or polypropylene. Compared with other elements, arsenic compounds are relatively stable. The main problem associated with storing solutions containing arsenic combinations is the limited stability of As(III) compounds, which are prone to rapid oxidation (complete oxidation at room temperature occurs within 24 h) [39, 40]. Compounds of As(III) are found in every living organism because they are intermediate products in the biomethylation of arsenic (V) acid derivatives. Oxidation has been demonstrated to occur with glutathione, following which the dihydroxy arsenate(III) anion undergoes transmethylation [41]. A standard method for stabilizing liquid samples is UV irradiation. A drawback of the method, however, is that it accelerates the process of oxidation. Another commonly used method is acidification with nitric acid (beneficial from the viewpoint of ICP-based assays). Nitric acid, however, is also an oxidizer and shifts the speciation equilibria towards compounds of As(V). On the other hand, hydrochloric acid, which does not possess oxidizing properties, gives rise to problems at the stage of detection because chloride is a source of chemical interference in MS using a detector equipped with quadrupole analyzer without a collision cell.

The process of oxidation of arsenic compounds is also observed in solutions containing iron and manganese ions, which readily mediate redox reactions [42]. One solution in this case is to introduce EDTA or another substance capable of creating complexes with ions of these metals [43]. Ligand-type compounds also prevent the formation of insoluble combinations of inorganic forms of arsenic with iron [44]. Increasing the stability of samples can be achieved using ascorbic acid (a reducer) in the environment of acids complexing iron and manganese ions [45], or by rinsing the solution with a neutral gas before tightly closing the vessel [46].

An important problem in the speciation analysis of arsenic is the above-mentioned co-precipitation of oxidized forms of arsenic with sparingly soluble compounds of iron or manganese, or the formation of iron and calcium arsenates [47]. The phenomenon is not observed in an acid environment (at pH levels below 2); the most stable solutions are achieved when phosphoric(V) acid at a concentration of 10 mM is used with EDTA addition. Solutions are stable for 4 days at room temperature in the presence of manganese and iron at a concentration of 100 mg L^{-1}. Reduction in temperature to 4 °C (but without freezing) and protection from light considerably prolong the period of sample storage to 28 days; after 3 months the change in the content of arsenic compounds does not exceed 10 % [48]. The procedure is required for all solutions containing iron, manganese, or calcium ions, particularly natural waters and soil extracts [49]. For the storage of aqueous solutions of samples of biological origin (e.g., yeast), it is generally sufficient to reduce the temperature to 4 °C and ensure protection from light. Arsenosugars, AsB, and arsenocholine (AsC) exhibit good stability, such that degradation products are not observed until after 9 months of storage (Fig. 12.1) [50] even at room temperature, provided the concentration of arsenic compounds is appropriately high (>0.5 mg mL^{-1}) [51].

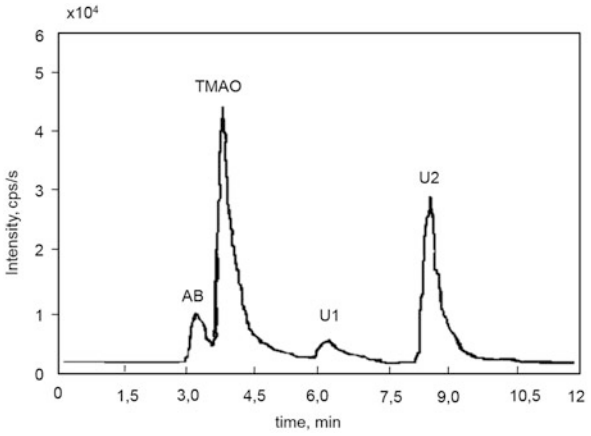

Fig. 12.1 HPLC ICPMS chromatogram of an extract from the standard reference material, after the extract was stored at 4 °C for 9 months, *AB* arsenobetaine, *TMAsO* trimethylarsine oxide, *U*1 and *U*2 unidentified arsenic species [50] (Reprinted with permission. Copyright (1994) American Chemical Society)

Most problems during storage are caused by complexes of arsenic and phytochelatins, which, during the freezing of the solution, are subject to decomposition triggered by the process of peptide oxidation. In this case, acidification and addition of complexing substances to samples are not viable options. The safest procedure is to rinse the solution with a neutral gas or add a substance with reducing properties [e.g., beta-mercaptoethanol, dithioerithreitol, or tris(2-carboxyethyl)phosphine)] to prevent the formation of sulfide bridges [52].

Knowing the stability of the compounds under study plays a key role in designing appropriate conditions for their storage, isolation, purification, and correct determination. The example of arsenic shows that despite many years of research aimed at determining the characteristics of its derivatives, there are still a multitude of problems, particularly for inexperienced analysts, even at the stage of stabilization of collected samples.

12.3 Determination of the Total Content of Elements

In many cases, speciation procedures must be preceded by obtaining information about the total content of a particular element in the analyzed material. In this way, it is possible to assess recovery during the extraction process or the degree of interaction of the compounds of the assayed element with the stationary phase during chromatographic separation. This involves examination of solutions obtained through wet mineralization, extraction, fractionation by centrifugation, and filtration and in solutions collected from chromatography columns and solid phase extraction (SPE). In assays determining total analyte content, spectroscopic techniques such as atomic absorption spectrometry (AAS) and OES are broadly used. They are sometimes preceded by a hydride generation module, which makes it possible to reduce the limit of detection from 50 to 1 ng mL^{-1}. This is related to the fact that many elements interfering with the arsenic signal create volatile

derivatives in other conditions, or fail to create them at all. These include Al (in ASA and OES) and Cr, Co, V, and Cd (in OES). Electrochemical methods such as adsorptive stripping voltammetry (ASV), adsorptive transfer stripping voltammetry (AdTSV), and potentiometric stripping voltammetry (PSA) still retain their importance [53, 54]. However, the most prominent method in trace analysis (although still limited by considerable equipment costs) is ICP MS. The technique offers good isotopic sensitivity, ensuring the specificity of assays. Its major disadvantage is interference, which, despite less frequent occurrence than in AAS and OES, can nevertheless be a source of serious errors.

ICP MS is associated with two types of interference, physical and isobaric [55]. The former arise at the stage of sample loading into the detector and, more specifically, have their origin in the efficiency of spraying, which depends on the density and viscosity of the solution and its surface tension. In contrast, isobaric interferences arise through the overlapping of signals generated by different ions (mono- or polyatomic) having the same *m*/*z* value (i.e., the same ratio of the mass of the ion to its charge) as the analyte signal. Interferences can be caused both by components of the plasma gas and constituents of the sample [56]. There are a variety of ways to eliminate the interfering influences in ICP MS systems with quadrupole analyzers. One method is the selection of another, non-interfering, isotope of the investigated element for monitoring purposes [57]. This method, nevertheless, is not suitable for monoisotopic elements (e.g., As, Co). When assaying elements of low atomic mass, it is possible to reduce the temperature of the plasma [58]. This solution, however, can compromise the sensitivity of the procedure because of the high ionization potential of some atoms (e.g., As).

Beneficial effects can be obtained through optimization of nebulizing gas flow [59] or the position of the burner in relation to the connecting module (interface). In this way, it is possible to markedly reduce the quantity of oxides, hydroxides, and multicharge ions with only a slight reduction in sensitivity [60]. This solution, however, is not practicable for arsenic (^{75}As) because of interference by ions formed from atoms of the plasma-generating gas (argon) and chlorine typically originating from the sample matrix ($^{40}Ar^{35}Cl^{+}$). Taking into account the fact that argon and chlorine are the main components, and arsenic occurs at trace level in analyzed material, results obtained in this manner can be inconsistent with reality. It is possible to reduce the effect of chlorine on analysis results by adding a small amount of methanol (1–2 %), but only if the examined solutions have low ionic strength. Moreover, the modification could also entail contamination of the system by carbon depositing on the interface cones [61].

In arsenic assays, one possible way to eliminate interference resulting from the isobaric ions referred to above is application of correction equations. The contribution of argon chloride to the value of the signal measured for the ^{75}As ion can be calculated because chlorine occurs as two isotopes, ^{35}Cl and ^{37}Cl. The latter forms the $^{40}Ar^{37}Cl^{+}$ ion in plasma (signal at *m*/*z* 77). The intensity of the signal makes it possible to assess the necessary correction of ion signal intensity at *m*/*z* 75 by applying an appropriate mathematical equation [24, 62]. The approach is only possible if the ratio between the As and Cl content in the sample does not exceed

100 [24, 62]. The applicability range of the correction equation cannot be extrapolated.

An effective method of eliminating isobaric interference is to use an instrument with a reaction/collision cell before the mass analyzer [63–65]. The chamber of the instrument is filled with gas, either (1) a neutral gas called a collision gas (e.g., helium [66, 67]) that, as a result of collisions, reduces the energy of interfering complex ions and thus prevents them from reaching the quadrupole analyzer, or triggers their disintegration; or (2) a reaction gas (methane [68], hydrogen [59, 69], ammonia) that, by reacting with $ArCl^+$, is responsible for the formation of new polyatomic ions with a different mass [57], for example:

$$^{40}Ar^{35}Cl + H_2 \rightarrow {}^{40}Ar^{1}H_2^+ + HCl \tag{12.1}$$

Methane, which is used as a reaction gas in arsenic assays, causes the formation of the $^{75}As^{12}C^{1}H^{1}H^+$ adduct (ion at *m*/*z* 89) offering better sensitivity of the method than the ^{75}As isotope [70].

If using an instrument with a collision cell, the introduction of a reaction gas elevates the risk of formation of polyatomic ions that can also interfere with the determination of investigated elements. In analyses of environmental materials, other elements (e.g., Ca [71, 72], Fe, and Cu) are also potential sources of major errors. Components of the instrument (e.g., nickel cones) are not neutral for analytical process. The most common errors include using an interfering element (e.g., germanium) as internal standard [63]. The interfering character of elements and their ions (affecting arsenic assays) descends in the following order [73]:

$$^{58}Ni^{16}O^{1}H^+ > {}^{60}Ni^{14}N^{1}H^+ > {}^{59}Co^{16}O^+ > {}^{74}Ge^{1}H^+ > {}^{58}Fe^{16}O^{1}H^+ > {}^{40}Ca^{35}Cl^+ > {}^{63}Cu^{12}C^+$$

An effective way to eliminate interferences caused by chlorine ions is to use chromatographic separation [9] before arsenic analysis by ICP MS [61]. Eluents include solutions containing phosphate ions or, less commonly, carbonate ions [74]. The technique can also be applied for controlling the occurrence of interfering influences. Figure 12.2 presents chromatograms of a water sample monitored concurrently at *m*/*z* 35 and 75. The first (Fig. 12.2a) demonstrates that the material contains a considerable amount of chloride ions. The second chromatogram (Fig. 12.2b) shows the absence of the interfering ion $^{40}Ar^{35}Cl^+$, demonstrating that it is not formed under optimized operating plasma conditions. If a complex ion cannot be eliminated, HPLC is a helpful technique that enables separation of chloride anions from arsenic compounds.

Despite the shortcomings outlined above, ICP MS is widely used for determining the total content of elements in a sample, thanks to its very good sensitivity. However, the full potential of the technique is generally realized only in laboratories with experienced staff.

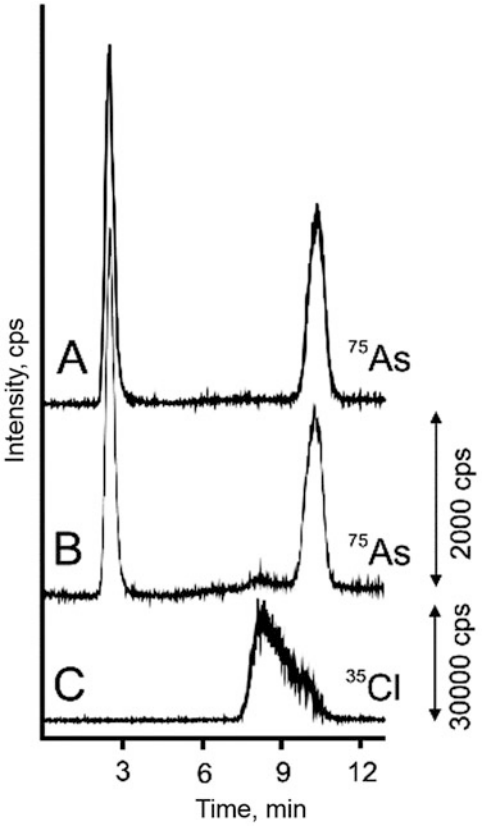

Fig. 12.2 ICP MS chromatograms of a water sample with (**a**) 10 ng mL^{-1} As(III) and As(V) without chloride ions, (**b**) 10 ng mL^{-1} As(III) and As (V) and 400 µg mL^{-1} chloride ions, (**c**) 400 µg mL^{-1} chloride ions; signal monitored at 35 and 75 *m/z* ($^{75}As^{+}$)

12.4 Extraction of Forms of Elements from Solid Materials and Concentration of Solutions

Extraction is the process of transferring a substance from a solid to a liquid phase or from a liquid to another liquid phase (immiscible with the former). From a practical viewpoint, the process can be achieved by leaching, which is transfer of compounds from a solid phase to a solution (solid–liquid extraction, SLE) or by extraction via direct (liquid–liquid extraction, LLE) or indirect (SPE or solid phase micro-extraction, SPME) transfer of a substance from one liquid phase to another [75]. The efficiency of the extraction process is expressed as the percentage of extraction, which takes into account the affinity of the investigated compounds for both phases. In practice, a commonly used concept is that of recovery, understood as the degree of transition of a substance from one phase to another, expressed as a percentage. There are multiple methods for determining recovery. They can be divided into two classes:

1. Based on assessing the quantity of an element in an extract in relation to its total quantity obtained by sample mineralization, or in relation to the overall quantity of an element in different extracts and in the mineralized post-extraction deposit
2. Based on assessing the recovery of different forms of the investigated element using the method of standard addition [76] or by analysis of a certified reference material (CRM) having a similar or identical matrix

In the latter case, it is advisable to use reference materials, if available, because the addition of an internal standard carries a risk that the degree and manner of binding between the compound and matrix are not accurately reproduced, leading to an overestimation of recovery. In the former case, however, the procedure is simple and problem-free from the analytical point of view. Recovery can be assessed for both leaching and extraction.

The process of leaching can progress in a variety of ways, depending on the type of extractants. The process can use the following extractants:

– Aqueous solutions of acids [77], alkalis [78], buffers, or compounds possessing strong oxidizing or reducing properties to obtain water-soluble compounds. This variant is usually long-term and incomplete, which is why the process is typically enhanced using ultrasound or by increasing the temperature (usually using microwaves) [79].
– Organic solvents, which enable the separation of polar organic or metalorganic compounds from the sample matrix. Solvent extraction is most commonly performed in a Soxhlet extractor [80], where the sample is in contact with cooled condensed solvent vapors, allowing extraction of volatile or thermally unstable compounds. The process can also be performed using a variant of the accelerated solvent extraction (ASE) technique, also referred to as pressurized liquid extraction (PLE), with a solvent under increased temperature and pressure [81]. In this case, leaching is performed in high pressure conditions (30–200 atm) and at a temperature of 50–200 °C [82].
– Solutions containing active enzymatic proteins (protease, lipase, trypsin, pepsin, prophase, or cellulase) or their mixtures, adjusted to the nature of the matrix of the solid material of biological origin [79, 83, 84]. The aim of the procedure is to break up proteins, polysaccharides, or fat chains and release the constituent amino acids, sugars, or short aliphatic chains. Enzymatic decomposition of the matrix can be considerably enhanced by application of ultrasound; the process can, for example, increase the efficiency of disintegration of cell walls in yeast and thus improve the recovery of selenium by as much as 20 % [85].
– Supercritical fluids, most commonly carbon(IV) oxide, occasionally modified by a small addition of a polar solvent (methanol, acetonitrile, or water). Supercritical fluid extraction (SFE) uses water as the most popular additive, because increasing the temperature from 50 to 400 °C at a pressure exceeding the critical level makes it possible to achieve transition of extractant from the subcritical to the supercritical state and leaching of the compounds in the order of polar to moderately polar [86].

Selecting the best medium for the leaching process depends on the properties of the analyzed compounds, the sample matrix, and the degree of chemical or physical bonding of the material's components. The level of hydrophobicity of the matrix significantly affects the effectiveness of extraction, even if substances released in the process exhibit high polarity. The breakup of the "grid" of the matrix by its dissolution or by physical, chemical, or enzymatic disintegration increases the likelihood of the investigated compound being efficiently transferred to the solution. The simplest method is to place a sample (e.g., of a plant) in liquid nitrogen (−78 °C) to destroy the cell wall structure or to add a surfactant such as sodium dodecyl sulfate (SDS) to locally alter the organization of phospholipids in the cell membrane, enabling release of hydrophobic proteins. Flavonoids, which are soluble in organic solvents and occur in plants of low thermal stability, are usually subjected to extraction in a Soxhlet extractor [87].

The leaching of arsenic forms is usually performed from soil or from tissues of plants or marine animals. The extraction of materials of biological origin typically involves methanol or mixtures of methanol and water. Solutions obtained by centrifugation and filtration are diluted with water and then loaded into an ion-exchange column. The method is applied for assay of arsenic acid salts and arsenosugars, even though they are more easily soluble in water than in methanol. This method of extraction into a solution involves transfer of a smaller quantity of toxic As(III) and As(V) salts than methylated derivatives and AsB or AsC, which are of markedly lower toxicity [88].

A higher methanol content in the extraction mixture enhances the effectiveness of extraction of organic arsenic derivatives without altering the effectiveness of release of As(III) and As(V) salts, and arsenosugars [89]. The duration of the process can be reduced by ultrasound treatment or pressurized extraction [90]. Multiple extraction does not improve the efficiency of the process; the quantity of arsenic remaining in the solid material depends solely on the volume of solvent retained in it (usually in the first cycle) and not on the number of repetitions of the leaching process [91].

Occasionally, the extraction of arsenic forms can be conducted using demineralized water, but only in cases involving the release of stable compounds in solutions of varying pH. It has been employed, for example, in comparative analysis of the hair of 1000-year-old mummies recovered from the Atacama Desert in Chile, and in analyses of hair collected from contemporary residents of India inhabiting areas contaminated with arsenic [92]. Similar contents of dimethylarsinic acid [DMA(V)] and monomethylarsonic acid [MMA(V)] were found in both materials under study.

Hot water extraction is not recommended for analyses investigating the speciation of arsenic in seafood, animal tissues, or bodily fluids focused on the detection of toxic methyl derivatives of arsenic(III) acid. These derivatives are intermediate products that exhibit low stability, particularly under in vitro conditions [93–97].

Because arsenic is chemically similar to phosphorus, it is capable of replacing that element in phospholipids, forming arsenolipids. A study of algae has found that as much as 50 % of the element occurs in the form of lipids extracted by means of

Table 12.2 Solutions used in sequential extraction

Leaching of metals from soil	Leaching of metals from plant
1 M $MgCl_2$ or CH_3COONH_4, pH 7 For metal ions binding to soil by ionic interactions	10 mM Tris-HCl, pH 7.4 For peptides from the vacuole and cytosol
1 M CH_3COONa or 0.05 M EDTA, pH 5 For metal ions in the form of carbonates or adsorbed on the surface of a hardly soluble soil matrix	2 % driselase or mixture of cellulase in 10 mM Tris-HCl, pH 7.4 For peptides and polysaccharides from the cell wall
0.1 M NH_2OH, pH 2 or 0.2 M $NH_4C_2O_4$, pH 3 For hardly soluble metal oxides (e.g., Mn, Fe, and Al), which are reduced	2 % SDS in 10 mM Tris-HCl, pH 7.4 For hydrophobic proteins
30 % H_2O_2 For metal ions strongly binding with organic compounds	10 mM ammonium acetate, pH 4.5 For organic acids such as citric acid and malic acid

nonpolar solvents. Reports on the identification of these compounds, however, are scarce [98]; for seafood, it is necessary to perform preliminary de-fatting of the sample with acetone, diethyl ether, or chloroform. The procedure makes it possible to obtain a homogeneous sample, but causes a loss of between 12 and 30 % of arsenic [99, 100]. Better recovery has been achieved using trypsin for protein breakdown in seafood [101–103].

Studies investigating the content of arsenic in the soil are generally based on extraction with demineralized water, enhanced by shaking or microwave treatment [104, 105]. The latter method has also been used for analysis of biological materials [106, 107]. An improvement in the stability of As(V) compounds in extracts has been obtained through the addition of phosphoric and ascorbic acids [105].

The differing stability of element forms in different solvents and aqueous solutions (from acidic to alkaline) sometimes allows sequential extraction. The chemical activity of extractants is gradually increased by elevating ionic strength, reducing pH, or introducing complexing agents. For analyses of arsenic forms, it is not recommended to modify leaching media with substances that have oxidizing or reducing properties. With appropriate selection of solvents, sequential extraction sometimes enables the reproduction of natural processes occurring in the environment. If the aim of an analysis is, for example, to determine the level of mobility/immobility of elements in the soil [108] or plants (Table 12.2), it is not always necessary to perform complete transfer of a particular element into solution (which is desirable for trace analysis).

Solutions obtained through leaching, or samples of water, must usually be concentrated before analysis. The most frequently used methods are lyophilization (concentration by the sublimation of water), evaporation of solvent under reduced pressure and temperature conditions, or accelerated evaporation in a stream of neutral gas. Each of these methods normally allows preservation of identity, even in the case of thermally unstable compounds. However, during distillation at a temperature above 120 °C, methylated derivatives of As(III) can be transformed into As(V) derivatives and degradation of other forms can be observed [109].

Considerably milder conditions are necessary for concentration using SPE, a process that is also available in a "miniaturized" version, SPME.

SPE was first developed by Zhang and initially used for the isolation of organic compounds from aqueous matrices [110]. The technique makes it possible to condense analytes in two stages: sorption on an appropriately selected solid phase, followed by desorption with small volumes of eluent [111]. SPE cartridges contain a variety of packing materials typical for liquid chromatography (polar, hydrophobic, and ion exchanging). Concentrated solutions obtained in this way can be loaded directly into an HPLC or gas chromatography (GC) column [75]. For materials of biological origin, good effects are obtained by combining SPE with leaching. Cartridges are filled with homogeneous solid material harvested from a tissue (usually of plant, fungal, or yeast origin) mixed with standard beds used in SPE. Such "matrix SPE" is most commonly used for isolating organic compounds [112]. The efficiency of extraction can be improved by placing a cartridge, previously filled with an appropriately selected eluent, in an ultrasonic washer. This method combines the stages of extraction and preliminary purification [113, 114]. Elements of process automation are obtained by combining matrix SPE with a PLE system [115].

A SPME kit consists of a "microsyringe" having a plunger with a fused silica fiber coated with an active material (sorbent) fixed at the end. The most common substances used for that purpose are polydimethylsiloxane, polyacrylate, polyimide, or packing material used in gas or liquid chromatography columns. The fiber with the active material can be immersed in the solution under study or introduced into the vapor accumulated above the liquid in a closed vessel. The method is used primarily in combination with GC. The heated dispenser of a chromatography device allows thermal desorption of compounds from the fiber and their direct transport into a GC column, which markedly reduces sample loss [116]. Nonvolatile substances, including metalorganic compounds, need derivatization to obtaining volatile derivatives [117].

A fiber coated with sorbent can be exchanged for a microsyringe containing hydrophobic solvent (single-drop microextraction, SDME). The syringe needle is placed in a polar solution of the sample, and a single drop of the hydrophobic solvent is pressed out. On completion of the process, the needle is withdrawn back into the container. In this extraction mode, analytes undergo absorption in solvent in a volume of 1–3 μl and can be introduced directly into the GC or HPLC injector [117, 118]. The method is considered complementary to SPME and also enables extraction of inorganic compounds (direct SDME [119, 120]).

Each of the methods described above allows rapid sample concentration, and can (and should) be employed in analyses of chemical compounds at the trace or ultratrace level.

Even as little as 20 years ago, arsenic compounds were mainly analyzed by LLE. Organic and inorganic forms were separated by arsenic reduction in its inorganic combinations, leading to formation of halogen metal acids or heteropoly acids extracted with toluene or trichloromethane. Organic arsenic was assayed as the difference between the total quantity and the quantity extracted by performing the

procedure outlined above [121]. As(III) assays omitted the stage of reduction [122]. The process was labor-intensive, nonselective, and rather inaccurate.

The application of SPE has partially eliminated the above problems, particularly in the case of arsenic concentration in water samples [123]. SPE delivers better selectivity than LLE because it can be used to sequentially elute compounds from the activated carbon bed, and to separate inorganic compounds of As(III) and As (V), as well as the phenyl (PAS) and dimethyl (DMA) derivatives of arsenic (V) acid [124]. The extraction process is short, which is why it is possible to directly connect the SPE module with the ICP MS detector [125]. Whenever modified silica is used, arsenic recovery is low (even below 50 %) owing to the formation of hydrogen bonds between the substances being separated and silanol groups [114, 126, 127].

The application of SPME for the release and enrichment of inorganic arsenic compounds is not suitable in selective assays of the As(III) and As(V) forms because they are transformed into volatile hydrides before the extraction stage [128, 129]. The same problem concerns single-drop extraction, which is performed in the headspace analysis mode, also after transforming arsenic into volatile hydrides [130]. Partial selectivity of operations can be achieved by controlling the acidity of the solution, appropriately selecting the type and concentration of the reducer, and using complexing reagents [131]. Although the approach requires appropriately selected assay conditions, it enables analyses to be performed at a content level of picograms per milliliter .

The techniques outlined above allow at least tenfold concentration of investigated compounds. Unfortunately, the procedure often entails concentration of the matrix, which is a potential source of noise in the detector. Even though removing the matrix improves selectivity, the ratio of the signal generated by the substance under study to the signal of the matrix (S/N), it can also upset the equilibrium existing between different forms in analyzed materials. Therefore, extracts should be assayed over the shortest time possible, which can be achieved with techniques that are chromatographically linked to sensitive detectors.

12.5 Determination and Identification of Element Forms: Application of Coupled Techniques

Coupled techniques are a basic tool in inorganic and bioinorganic speciation analysis. Their status results mainly from the possibility of using sensitive and specific detectors, whose advantages can be fully exploited by coupling to high-performance separation techniques, mainly GC, HPLC, CE, and supercritical fluid chromatography (SFC).

12.5.1 Gas Chromatography

GC is employed for the separation of chemical compounds of high and medium volatility. The effectiveness of the process is conditional on differences in boiling points between individual substances and the extent of their interactions with the stationary phase (in packed columns) The basic precondition for GC is the volatility of analytes at temperatures of up to 300 °C. The precondition is met by alkyl derivatives of metals and metalloids, and some amino acids. The majority of metalorganic compounds that are significant in environmental analyses (alkyl or phenyl derivatives) must be subjected to derivatization, resulting in the formation of a volatile derivative. Aside from $NaBH_4$, which is used for the formation of volatile hydrides, such reactions mainly use tetraalkyl borates [132]: $NaBEt_4$ to obtain ethyl derivatives of mercury compounds; $NaBPr_4$ for propyl derivatives of tin, mercury, and lead (in aqueous environments); and $NaBBu_4$ for butyl derivatives in nonaqueous environments in the presence of Grignard reagent [133]. Alkylation reactions are not typically quantitative as a result of concurrent transmethylation. The secondary process, however, can be controlled by enriching the sample with a standard containing a stable isotope [134].

The application of tetrapropylborate makes it possible to simultaneously assay Sn, Hg, and Pb compounds [135]. Esterification of the carboxyl group of selenomethionine with 2-propanol after acylation of the amine group [136] causes a slight modification in the molecule's structure, making it possible to verify its identification by molecule-specific GC MS.

The most volatile metalorganic compounds or hydrides (at a temperature below 100 °C) are separated in columns packed under isocratic elution conditions. This mode does not offer good resolution, which rules out separation of forms of several metals. An excessive quantity of organic compounds introduced simultaneously into the plasma (in the GC ICP MS variant) decreases its energy, which sometimes causes the plasma to be extinguished.

Capillary GC gives better resolution and allows simultaneous analysis of alkyl derivatives of a number of metals. Such columns, however, are easily "overloaded" and their application necessitates using smaller analyte quantities than in packed columns. The sensitivity of analysis is also lower as a result of the higher relative content of oxygen and, possibly, aerosol from the connector in the stream leaving the column and entering the plasma. An improvement in the sensitivity and resolution of capillary GC, as well as a considerable reduction in analysis time, can be achieved by using short multicapillary columns. A bundle of capillaries enables separation of larger quantities of analyte. In addition, the total carrier gas stream requires only minimal dilution. Short analysis time combined with the possibility of applying high carrier gas flow rates enables isothermal separation of compounds with markedly different boiling points. This, in turn, reduces the cost of equipment and, ultimately, the cost of analysis [137].

In practice, speciation analysis of metalorganic compounds is conducted using three coupled systems: GC with microwave-induced plasma optical emission

spectrometry (MIP OES), ICP MS, and electron ionization mass spectrometry (EI MS). Methyl derivatives of mercury can also be assayed with AAS and AFS spectrometers as detectors, and alkyl derivatives of tin using a flame photometric detector (FDP), on account of their good sensitivity. Multielement analyses should preferably be conducted with an ICP MS spectrometer, especially if it is equipped with a collision cell enhancing the selectivity of the instrument, particularly in assays involving light elements (e.g., S, F, Cl) previously examined by MIP OES. ICP MS is a technique whose sensitivity allows detection of elements at a level of 1 fg. Furthermore, the isotopic specificity of the method decreases the matrix effect. The preservation of original isotope ratios makes it possible to perform analyses using isotope dilution of the sample or eluate stream. It offers the best quantification precision and does not require using standards. The precision of assays can also be improved by reducing detection time. For this, gas chromatograms are coupled not only to MS detectors equipped with sequential quadrupole filters but also to time-of-flight (TOF) analyzers (quasi-simultaneous detection) or sector analyzers with multichannel detectors (simultaneous detection). The improvement in assay precision achieved in this manner is an effect of a decrease or exclusion of the impact exerted by the conditions of ionization and ion extraction on the intensity of signals generated by different isotopes, and an increase in the number of measurement points defining the short (several seconds) chromatographic peak.

Calibration in ICP MS, in methods based on recording the intensity of signals generated by different isotopes, can be carried out by the following methods:

1. Isotope ratio, based on a comparison of the ratio between the intensity of signals generated by selected isotopes of a particular element in the sample to the intensity of the added standard. An external standard is routinely added in a stream of auxiliary liquid.
2. Isotope dilution, based on adding to the sample the assayed form of the element under study, enriched with its stable isotope. Controlling the state of the standard substance is possible by incorporating into the analytical process the chemical transformations of the investigated form occurring during extraction, derivatization, and ionization in plasma of varying power.

The method of isotope ratio has been employed predominantly in assays of volatile compounds of lead, tin, and selenium. The applicability of the method of isotope dilution depends on the availability of isotopically enriched standard forms. To date, it has been utilized for assaying methyl derivatives of mercury and butyl derivatives of tin. The effectiveness of the former method, which is more widespread, is dependent to a large extent on the quality of the module connecting the gas chromatograph to the ICP MS spectrometer [138].

In a standard ICP MS spectrometer, a sample is loaded into the ionization (plasma) zone in the form of an aerosol at room temperature. The connector between the chromatograph and the spectrometer must prevent the condensation of vapors of separated compounds. Usually, it has the form of a resistance-heated steel capillary connected to detectors in the following modes:

1. Directly: This ensures maintenance of resolution and sensitivity as a result of the absence of dead volume in the system [139].
2. Via a liquid dispenser: Eluate from the liquid chromatograph is mixed with auxiliary liquid from the dispenser, and aerosol is formed. The method is used for flow calibration of the isotope ratio because standards of stable isotopes are most typically used as aqueous solutions. Dilution of the eluate stream lowers the sensitivity of the method, but does not lead to a decrease in resolution caused by the dead volume of the connector [140].
3. Via a flow chamber: The eluate stream is enriched with an element standard diffusing through the membrane from the diffusion chamber [141].

Analysis of samples introduced into a GC column in the form of solutions in organic solvents requires addition of oxygen to the eluate stream to achieve combustion of the organic matrix, accompanied by formation of carbon oxides. The operation prevents accumulation of carbon in the system (e.g., on the cones) and in this way avoids changes in sensitivity and eliminates interference resulting from the presence of solvent [142].

The main disadvantage of GC in speciation analysis is the need for derivatization, leading to the formation of volatile derivatives of metals (e.g., mercury). Because the arising forms are toxic, appropriate means of automatic sample preparation and column loading must be ensured. One of the available solutions is the application of a flow reactor for the alkylation or reduction, accompanied by formation of volatile Pb(II) and Hg(II) hydrides and compounds of Se(IV) and As (III). The emerging forms are transferred via a water wash to a capillary where they are condensed by cryogenic focusing (−100 °C) and then, following release, they are separated in a multicapillary column. The above approach reduces the duration of the analytical procedure from several hours to several minutes [143].

Arsenic is one of the chemical elements whose speciation analysis is rarely performed by GC. Arsenic is a monoisotopic element, and this precludes determination of the isotope ratio. It also has a limited capacity for derivatization. The process aimed at the formation of volatile hydrides from its inorganic compounds [144] eliminates the possibility of gaining information about the original form. The derivatization of some arsenoorganic forms with L-cysteine [145] requires the concentration of separated substances in a capillary by cryogenic focusing prior to detection by MS, AAS, AFS, or MIP OES. The applicability of GC in arsenic speciation studies is low because of the polarity of its forms. This feature, however, ensures good solubility in water or in a water/methanol mixture, which is why the most appropriate separation method is liquid chromatography.

12.5.2 Liquid Chromatography

Liquid chromatography is a technique for the separation of nonvolatile and high molecular mass compounds without their derivatization. It enables selective

separation of analytes as a result of their varying affinity to the stationary and mobile phases. The effectiveness of the process can be adjusted through appropriate selection of the composition of the mobile phase or a change in the stationary phase.

Speciation analysis most commonly utilizes the following techniques:

Size exclusion chromatography (SEC, also known as gel permeation chromatography) is a method of separating compounds of different molecular masses and sizes. Because steric interactions between analytes and the stationary phase are relatively weak, unstable forms of metals can be separated from more stable complexes and from adducts stabilized by ionic interactions. Unfortunately, the process of sorption and ionic interactions between the investigated substances and the stationary phase can decrease metal recovery by as much as 50 %; these interactions are also responsible for the instability of retention times [146]. The separation can be performed both in the aqueous environment and in the presence of organic solvents. Because the technique is not selective, it is utilized primarily as the first stage of multidimensional chromatography [147].

Ion chromatography (IC) allows the separation of substances in the form of ions, chiefly in aqueous solutions. Mobile phases used in the technique contain relatively large amounts of salts stabilizing the pH and determining the sequence of analyte retention. They enable the separation of only cations or only anions; the ions that are not separated by a selected phase (cationic or anionic) are eluted in the column dead volume. As the next step, they can be loaded into appropriate ion-exchange columns in the second chromatographic dimension [148].

Reversed phase liquid chromatography (RPLC) allows the separation of analytes with different hydrophobicity and polarity characteristics. It has good selectivity; mobile phases used in the technique contain organic solvents and small amounts of inorganic salts [149]. The effectiveness of the process depends on the hydrophobicity of the separated analytes. Charged substances must first be transformed into neutral derivatives (e.g., by adding appropriate anti-ions into the mobile phase).

Ion-pair liquid chromatography (IPLC) is a variant of RPLC and ensures good selectivity of the process, provided that an appropriately low concentration of inorganic salts is retained in the mobile phase and that they are competitive to the counter-ion or modify the stationary phase [150]. Methods based on this technique are not recommended for routine analyses because of their nonrepeatability.

Normal phase liquid chromatography (NPLC) and *hydrophilic interaction liquid chromatography* (HILIC) enable separation on the basis of differences in polarity between analytes engaged in ionic or dipole interactions, or through hydrogen bonds, with the polar centers of the stationary phase. The possibility of using polar organic solvents, water, and their mixtures, together with volatile salts for pH control [151], makes HILIC a multipurpose technique for separation of compounds that are too polar for separation by RPLC.

In trace speciation analysis, liquid chromatography is most typically combined with sensitive MS techniques. Routine assays of metal forms are usually performed using coupled HPLC ICP MS systems, obtained by connecting the outlet of the chromatography column with a dispenser using a short hose (SEC, IC, IPLC, and

HILIC ICP MS). If the mobile phase contains an organic solvent (as in RPLC), it is necessary to cool down the aerosol, carry off oxygen for burning carbon and forming carbon oxides, and use cones made from a platinum-enriched alloy [152]. Often, it is also necessary to replace the spray chamber with one having a smaller dead volume, in order to enhance resolution. Another drawback of the RPLC ICP MS combination is the inferior repeatability of results in gradient elution, resulting from the fact that the content of organic solvent in the eluent increases over time. Routine analyses are normally conducted using the IC ICP MS combination, and screening analyses conducted using SEC ICP MS [153]. Basic research utilizes all combinations, depending on the type of compounds being analyzed and on their properties. On account of the possibility of recording metal isotopes by ICP MS and, increasingly frequently, in order to ensure a greater precision of assays, the method of isotope dilution is employed.

Before embarking on quantitative analysis, it is necessary to verify the identification of the compound under study by comparing its retention time with the value recorded for a standard. Verification can be performed by adding an internal standard, comparing the retention time with the retention time of the standard recorded by an alternative chromatographic method, or using a selective detection mode. The procedures are usually carried out in a HPLC ESI MS system, coupling liquid chromatography to ESI MS. The presence of a volatile organic solvent in this equipment configuration improves the efficiency of the process, hence, ESI MS is typically combined with RPLC [154]. Such combination has found a range of applications, for example, in identification of arsenic metabolites in plant and animal tissues. It has allowed the determination of the structure of arsenic complexes with phytochelatins (PCs) and mixed complexes with glutathione [$AsPC_3$, $GSAsPC_2$, $As(PC_2)_2$] [155] thanks to the possibility of separating peptides and their stable complexes in chromatographic conditions appropriate for ESI ionization conditions (the mobile phase contains an organic solvent and trifluoroacetic acid). Verifying the identification of compounds separated by IC ICP MS is not as straightforward because the process of electrospray ionization does not tolerate high amounts of salt in the eluent. In this case, it is often necessary to collect the eluate fraction from the IC column and desalinate it before placing it in the detector [156, 157].

An often disregarded parameter, which is nevertheless important for the assessment of the performance of the system, is chromatographic peak purity. It is assessed by measuring the ratio between the intensities of signals obtained for two characteristic peaks from the spectrum (e.g., $\lambda_{1max}/\lambda_{2max}$ or $m_1/z_1/m_2/z_2$). The ratio between the intensities of these signals within a pure chromatographic peak should be constant. Chromatographic software makes it possible to accurately and quickly assess the purity of a chromatographic peak, which should be checked during the analysis of every consecutive sample.

Liquid chromatography, a seemingly simpler procedure than GC in the speciation analysis of arsenic because it does not require the derivatization stage, also generates a number of potential errors. The selection of chromatographic method involves preliminary evaluation of the solubility of compounds to be separated. The

majority of known arsenic compounds are polar in nature, which facilitates the process of ionization during the detection stage and, at the same time, clearly implies the application of ion-exchange chromatography. These compounds are usually weak acids and easily undergo deprotonation. The tendency decreases in the case of As(V) compounds for organic derivatives [the pK_1 of arsenic(V) acid is 2.2; in contrast, the pK_1 of MMA rises to 3.6]. The acidic properties of arsenic(III) acid are markedly weaker (with the pK_1 of 9.22) and are more pronounced in methyl derivatives (the pK of DMA(III) is 6.2). The dissociation constants for arsenic compounds increase from 2.2 to 12.2. The broad range of pK values stems from the fact that they are polyprotic acids. This property of arsenic forms is significant because the degree of deprotonation of a compound under study affects the strength of its interactions with the alkaline stationary phase (i.e., the retention and selectivity of the method) [158, 159]. The pH of the mobile phase needs to be such that the separated compounds are ionized at a minimum efficiency of 90 % (p$K \pm 1$). An excessive affinity of the substance for the stationary phase makes it necessary to use a mobile phase of higher ionic strength. Anion-exchange chromatography of arsenic compounds usually utilizes weak ion exchangers (tertiary amines) and mobile phases with a pH of 6.0 or 8.5, adjusted by means of tetraethylammonium hydroxide [160], phosphate buffer, or carbonate buffer [161]. The choice is made with awareness that the environment is not adequately alkaline for the ionization of arsenic(III) acid; however, it prevents loss of other forms through precipitation or co-precipitation. Under such conditions, arsenic(III) acid can be eluted in the column dead volume along with compounds that, as a result of their alkaline properties, are not charged (e.g., AsB, AsC, arsenosugar B).

Anion-exchange chromatography is an effective technique for analyzing groundwater; however, its potential is often insufficient for the separation of compounds in samples of surface waters. Analyses of materials of biological origin should also be performed by cation-exchange chromatography because a number of arsenic compounds undergo protonation as a result of the As=O group present in their structure (AsB, AsC, some arsenosugars). The process is performed in a solution with pH of ca. 3. Under these conditions, arsenic(V) acid and arsenosugars A and C do not undergo protonation and are eluted in the column dead volume. Additionally, because the pH of the mobile phase is close to the pK of many compounds, their chromatographic peaks are relatively broad, nonsymmetrical (with a large tail), and occasionally even double. Resolution achieved in cation-exchange chromatography is inferior to that in anion-exchange chromatography. Nevertheless, the technique is useful, for example for assaying nontoxic AsB, which is found in large quantities in oysters and other marine organisms. Determining the content of these substance makes it possible to prepare a mass balance of arsenic compounds and confirm a small proportion of inorganic forms of this metalloid in its speciation.

The range of HPLC applicability can be extended in IPLC by using a mixture of malonic acid and tetramethylammonium hydroxide as counter-ions for protonated and deprotonated arsenic compounds. The technique offers the best resolution; however, it is associated with considerable measurement uncertainty because the

stationary phase is modified over time [162]. Another drawback is the risk of co-elution of arising ion pairs with neutral compounds [e.g., MMA(V) with arsenic(V) acid], which occurs because it is not possible to employ gradient elution (the equilibration time is too long) [163]. In actual fact, none of the techniques presented above is universal in nature, and none of them allows the separation of even ten arsenic compounds with different acidic and alkaline properties. Consequently, it is necessary to use several techniques, particularly if the materials under analysis have a complex biological matrix. One example is the assay of carcinogenic arsenic(III) acid, which has been observed to co-elute with the following:

- DMA, AsB, arsenosugar B, and tetramethyl arsonium ion in anion-exchange chromatography (recommended for analyses of water and marine plants containing multiple arsenosugars)
- Arsenosugars A, C, and D in cation-exchange chromatography (recommended for analyzing urine and seafood)
- AsC in IPLC (recommended as a reference method)
- AsC and DMA(III) in SEC (recommended for the separation of high molecular mass compounds)

Selecting a speciation analysis method that is best suited to particular needs requires careful verification of its selectivity. It is rarely possible to apply a direct approach based on the analysis of standard substance mixtures. This is evident in view of the fact that although over 20 arsenosugars have been identified to date, only a few are commercially available. Considering the circumstances, indirect methods are recommended, based on the assessment of purity of chromatographic peaks. There are two main variants of such assays. In the simpler variant, the gradient step during elution is prolonged and attenuated, and checks are performed to determine whether the peaks split. In the more time-consuming variant, fractions corresponding to different peaks are collected from the column, concentrated, and separated by an alternative chromatographic method. In this case, the process can be automated, and is particularly suitable for analyses of extracts harvested from biological tissues because it offers better assay accuracy and sensitivity as a result of elimination of the matrix effect.

An especially challenging task is maintaining the selectivity of the method for separation of compounds whose elution time is very short, close to the dead time. In such cases, it is necessary to perform a preliminary review of the planned chromatographic conditions, including the composition of the analyzed material. For example, a typical eluent employed in anion-exchange chromatography (with pH of 8.5) is intended to facilitate the dissociation of separated compounds. Neglecting the time necessary to achieve acid/base equilibrium of substances loaded into the column in a neutral solution can result in their elution in the dead volume. The phenomenon is observed, for example, for MMA(V), whose consecutive dissociation constants are pK_1 3.6 and pK_2 8.22 [164].

A reduction in retention times can also be observed in analyses of solutions of high ionic strength. If gradient elution is initiated with a phosphate buffer with a concentration of, for example, 1 mM, and the content of sodium chloride is 0.1–

1.0 M, the compounds being separated do not sufficiently interact with the stationary phase. The problem occurs most commonly in analyses of urine containing sodium chloride at a concentration exceeding 100 mM. Salinity of analyzed solutions is also problematic in RPLC (because of solvation of the investigated compounds) and in HILIC (as a result of competitive interactions with the stationary phase). These unfavorable phenomena often require salt removal. The procedure can be performed by SPE, among other methods [165].

Another factor adversely affecting resolution is the common anionic surfactant SDS. SDS is often present in extracts of biological materials because it is used for the transfer of hydrophobic proteins into aqueous solutions. It is also employed for protein release via gel electrophoresis. In some cases, however, it can be intentionally added to the mobile phase (in micellar liquid chromatography), making it possible to separate arsenic-binding peptides from inorganic forms of arsenic. In this variant, SDS also serves an additional role as an agent enabling the separation of arsenic(V) acid and sodium chloride, which is why no isobaric interferences characteristic for ICP MS are observed in the detection module [166].

If the analytes fail to interact with the stationary phase sufficiently strongly, they are eluted close to the dead time. In contrast, if the interactions are excessively strong, they are not completely removed from the column. The scale of the phenomenon can most easily be determined by assessing the recovery of the investigated compound from the column. Recovery is understood as the ratio of the quantity of eluted substance to the quantity of the substance loaded into the column. Knowing the value of recovery makes it possible to assess whether the conditions of chromatographic separation are correctly selected for the group of compounds under analysis, and determine whether other unknown substances are sorbed on the surface of the stationary phase. If the recovery of a particular element is found to be low, there are grounds to assume that some of its forms are sorbed on the bed in a permanent manner and their elution may only be possible after a change of eluent (pH). It is a result of studies of this type that, for example, a previously unknown arsenic compound was detected in certified reference materials [167], and another new compound (2-dimethylarsinothioyl acetic acid) was found in sheep urine [168].

Low column recovery can also be caused by an excessively large surface area of the stationary phase in relation to the quantity of analyte, which is a common tendency in trace analysis. For example, the recovery of arsenic in the separation of arsenic(V) acid, its methylated derivatives MMA, DMA, trimethylarsine oxide (TMAO), and arsenic(III) acid was 101–104 % in extracts from frog tissue in which arsenic was found at the milligram per kilogram level, and only 20 % in extracts from fish tissue containing arsenic in nanogram per kilogram amounts [169].

During analyses of materials containing analytes in markedly different amounts it is necessary to correctly plot curves for relatively narrow concentration ranges. Such “multilevel calibration” is a way of increasing assay accuracy because the slope of the curve increases with decreasing assayed amounts of the element.

For compounds exhibiting stronger interactions with the stationary phase, recovery can be improved by changing the strength of the eluent and maintaining gradient elution, which ensures good resolution. Potentially effective solutions in ion chromatography include changing the pH of the eluent, which weakens analyte interactions with the stationary phase, and modification of functional groups. An important process optimization criterion, however, is preventing the emergence of forms that strongly sorb on the bed [e.g., polyprotic anions formed by MMA(V)] [170] or forms that are sparingly soluble. Inappropriate conditions not only prolong retention time but also decrease recovery and increase the slope of standard curves. Another common finding is the broadening of peaks, especially in isocratic elution, which hinders integration. Studies of arsenic speciation require standard substances because determining the quantity of one form by referring the surface area of its signal to the surface area measured for another standard is a source of major errors.

The efficiency of elution of investigated compounds can sometimes increase with the number of analyses performed using a given column. In such cases, the same quantities of analytes produce larger peak surface areas. The tendency results from progressive modification of the stationary phase by the sorbed analyte; another effect can be the emergence of peaks of investigated compounds during blind sample analysis. The effect of the modification cannot be completely eliminated, even by prolonged washing of the bed, and results are affected by a positive error [171]. Similar errors can arise from the matrix effect; however, this can be detected relatively easily by analyzing samples at various degrees of dilution.

The described solutions to problems involved in the separation of element forms by liquid chromatography can create the impression that the technique readily provides accurate and precise results. This, however, is only partially true because the discussion above is focused on separation, disregarding all other stages involved in the process of speciation analysis. A comprehensive overview of other stages goes beyond the scope of this study; however, it seems worthwhile to briefly reflect on their importance for test results. As an example, we discuss interlaboratory studies of a homogeneous oyster powder (designed as a potential reference material) with AsB at a level of ca. 30 mg kg^{-1} as the main arsenic-containing analyte [172]. The basic analytical problems (except for unidentified issues) were found to be rapid photooxidation of AsB and co-elution of previously unknown forms of arsenic with AsB. The assay of other arsenic compounds was also burdened with large errors; for example, arsenic(III) acid was oxidized, and arsenic(V) acid was present below the limit of detection of most methods in use. AsC was identified and assayed in just one laboratory, and two types of arsenosugars were detected in several laboratories. There was no consensus as to the quantity of arsenic in the post-extraction deposit, even though the total content of arsenic in the mineralized material was certified.

The example clearly demonstrates the scale of difficulty involved in speciation analysis, and the fact that it requires a case-by-case approach. A helpful tool in the process is a list of typical errors (Table 12.3). Each time, however, one should be aware that the procedures are extremely complex, the stability of compounds is a source of multiple problems, and the quality of separation and assaying depends to a

Table 12.3 Typical errors in speciation analysis

Sample preparation and storage
Collecting an insufficient quantity of material (e.g., below the accuracy of the balance or the level of homogeneity)
Inappropriate methods of cleaning and conditioning vessels used for sample storage or sample transfer into the solution
Introducing excessive amounts of standards into the samples, which disturbs the equilibria in the material as a result of physicochemical modification of the nature of the matrix
Introducing an internal standard that interacts with the investigated compounds (via complexing, formation of ion pairs, oxidation–reduction reaction, addition or hydrolysis reactions)
Conducting extraction in uncontrolled pH conditions, or under elevated pressure and temperature, triggering processes of hydrolysis or alkylation
Failure to remove from the biological material active enzymatic proteins causing uncontrolled decomposition of compounds
Storing samples in inappropriate conditions (light, temperature, air access)
Measurements by ICP MS
Evaluating the recovery of the investigated element on the basis of addition of an inorganic standard rather than the proper form
Using an inappropriate correction equation
Adding an internal standard that interferes or is interfered with by the investigated element
Failure to consider the influence of the matrix on the quality of assays (i.e., failure to perform a preliminary semiquantitative analysis of the investigated material to determine the content of macroelements)
Adding interference-generating substances such as hydrochloric acid or chloric(VII) acid to samples
Separation and assaying by HPLC ICP MS
Failure to adjust the resolution of a method designed for investigation of the required number of forms (insufficiently high selectivity, unverified by analysis of standard substances or application of alternative chromatographic methods)
Failure to check the matrix effect by analyzing samples at various degrees of dilution
Lack of control of mobile phase temperature in ion-pair chromatography
Inadequate control of mobile phase pH
Failure to control retention times of interfering compounds
Incorrect plotting of calibration curves using a generally available substance rather than the form being assayed as a standard
Failure to check recovery of the element from the chromatography column
Using standard curves prepared over a broad dynamic range of concentrations rather than multilevel lines
Failure to control the purity of chromatographic peaks
Verification of compound identification on the basis of an excessively large addition of the standard

major extent on the equipment available in laboratories and on the advancement of packing and modification of beds in HPLC columns. Also, familiarity with the current state of knowledge on the speciation of an element of interest provides the best guidelines for resolving any arising issues.

12.5.3 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is a hybrid of liquid and gas chromatography that retains most of their advantages. The selectivity of the technique depends primarily on the type of bed used (usually, it is the typical stationary phase of liquid chromatography). The mobile phase is usually chemically neutral supercritical carbon(IV) oxide [173], which ensures that the method's efficiency is close to that obtained by GC. The investigated compounds do not need to be volatile, but they should be sufficiently polar to be soluble in water or methanol. It is possible to omit the process of derivatization, which is normally necessary in GC. In combinations of SFC with ICP MS, the main task of the connecting module is to ensure an appropriate temperature, preventing phase transformation of the mobile phase prior to delivery into the plasma [174]. It is necessary to split the eluate stream because of an increase in volume of the supercritical liquid accompanying the rise in temperature [175]. Stream splitting can be performed before or in the burner. In the former case, it is easier to control the temperature of the mobile phase; however, loss of heated eluate is larger. The latter case usually provides better sensitivity, but assay precision is worse. SFC coupled to ICP MS is rarely used for routine analyses, but represents an attractive alternative to GC and HPLC in basic research of low molecular weight compounds. It has an established status in speciation analysis of metalorganic alkyl derivatives of tin [176], lead [177], antimony, mercury, and arsenic [178].

12.5.4 Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique for separating ionized compounds in an (usually) aqueous environment. Compounds migrate at different velocities along the capillary made of fused silica in the electric field. Their velocity depends on electrophoretic mobility, which, in a large simplification, is proportional to the mass-to-charge ratio (m/z) of the ion. The most commonly used variant of the technique is capillary zone electrophoresis (CZE), in which ions move along with electroosmotic flow towards the negatively or positively (reverse CZE) charged electrode [179]. The quality of the separation process is determined by the type of electrolyte, which ensures an appropriate intensity of the ion stream providing the "pumping" power for analyte ions. Electrolytes used in the technique are buffers that are also responsible for maintaining the pH of the solution at a level ensuring that analytes are present in an ionized form. CE can be combined with ICP MS via a module that closes the electric circuit and replenishes the eluate stream from the capillary, so that its size is optimally suited to the spray conditions in ICP [180]. A combination of this type is often employed in analyses of arsenic compounds because it allows simultaneous separation of negatively and positively charged compounds. An additional advantage of CE is that a small sample (ca. 30 nL) is

sufficient for analysis. Unfortunately, however, this also reduces the sensitivity of the method. Arsenic compounds, most typically arsenic(III) and arsenic(V) acids, as well as MMA and DMA, in water are usually separated in a weakly alkaline environment in the presence of a cationic surfactant, which is intended to reverse the charge on the surface of the capillary and thus reverse the electroosmotic flow [181, 182]. The number of separated forms is dependent mainly on the quality of the module connecting CE with ICP MS, in which the eluate stream is replenished. Using a connector allowing mild suction of the auxiliary liquid makes it possible to maintain the piston flow from the capillary, which also enables detection of AsB, AsC, and phenylarsonic(III) acid [183]. The above equipment configuration has been successfully employed in the speciation analysis of arsenic in drinking water [184], urine, and soil extracts [185].

The combination of CE with ICP MS is technically complicated and prone to capillary blocking in the microsprayer, which diminishes the precision of assays. Another difficulty is the fact that the capillary needs to be led outside the system, which increases the temperature inside the capillary. These operational problems restrict the applicability of the system for basic research (e.g., interactions of inorganic arsenic compounds with histidine [186]) and exclude it from routine analyses.

12.6 Summary

Contemporary speciation analysis is developing in two main directions: the search for new forms of elements and introduction of the principles of modern chemical metrology into day-to-day practice. Activities encompassed by the former area are qualitative, aimed at the release and identification of often previously undescribed substances involved in living processes that have not been clarified to date. The other area, which requires the introduction of good laboratory practice, comprises routine analyses (i.e., assaying defined element forms) in real-life materials. Although the importance of correct interpretation of quantitative results seems obvious today, it did not always enjoy the status it has today.

Quantitative speciation analyses have been dominated by the coupled HPLC/GC ICP MS techniques on account of the good separation, sensitivity, and isotopic specificity of detector response. The same status in qualitative analysis is held by ESI MS coupled with RPLC and, increasingly, with HILIC. Sometimes, it becomes an alternative system in quantitative analysis for assays involving larger quantities of investigated compounds.

Speciation analysis procedures are complicated because they examine a multitude of element forms that demonstrate different physicochemical properties in a broad variety of environmental materials, which are characterized by different states of matter, origin, and analyte content.

Analytical processes of such complexity require careful design that takes into account all stages that could potentially be sources of errors, from the collection of

representative samples to the correct processing of results and assessment of the uncertainty margin. Only full utilization of available expertise and the current state of knowledge can give speciation analysis a major place in the development of bioinorganic chemistry, in which it should be applied as a basic study tool.

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Chapter 13
Quantification of Noble Metals in Biological and Environmental Samples

Maria Balcerzak

13.1 Introduction

Noble metals consist of six elements from the eighth group of the periodic table: ruthenium (Ru), rhodium (Rh), palladium (Pd), osmium (Os), iridium (Ir), platinum (Pt) (also known as platinum group metals [PGMs]), and gold (Au). Attractive physical and chemical properties of the metals, such as exceptional stability, hardness, malleability, electrical resistance, inertness to chemical attacks, and excellent catalytic activity, have resulted in their wide application, for example, as catalysts in various chemical processes; in autocatalysts (catalytic converters for vehicles); in electrical, electronic, and glass industries; in jewelry and investment [1]. PGMs are extensively used as the components of automobile catalysts (50.4 % of all uses in 2012 [2]). Confirmed release of ultratraces of these metals during vehicle operation as a result of thermal and mechanical abrasion of the catalysts is their source in the environment [2–10]. Medical application of some noble metal compounds is the second (after autocatalysts) anthropogenic source of these metals in the environment [11–13]. Since 1978 (date of the introduction of cisplatin, *cis*-diamminedichloroplatinum(II), to chemotherapy as an effective anticancer agent), numerous different complexes of platinum and other noble metal compounds have been extensively examined for anticancer activity [14–26]. Some of the second-generation compounds of Pt (carboplatin and oxaliplatin) have received worldwide approval for routine medical treatment, and some (lobaplatin, nedaplatin, and heptaplatin) are locally approved (in China, Japan, and South Korea, respectively). Cisplatin is still a leading drug, being used in 50–70 % of all anticancer chemotherapeutic treatments [17, 19]. There are numerous other metal compounds currently under clinical trial, and some Ru-based complexes seem promising because

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of improved antitumor activity and fewer side effects compared with cisplatin [15–18, 21–23, 25, 26].

Evaluation of the environmental impact and human exposure to PGMs requires examination of a large variety of samples of different matrices and complexity for traces of the metals, as well as investigation of biotransformation and bioaccumulation processes occurring in various environmental compartments and in living organisms. Transformation processes that can occur under the action of various oxidizing and complexing agents can result in the conversion of metals into more soluble and bioavailable species. Bioaccumulation can lead to elevated levels of these metals in some materials and increased potential health risk. Data on the sensitizing and allergenic effects of platinum metal salts on workers observed under chronic exposure (e.g., during production of catalysts) [27] show that the toxic effects of Pt and Pd on cellular systems are comparable to those of Cd(II) and Cr (VI) [28]. The tendency for bioaccumulation of PGMs in the environment pose questions about the possible health risk generated by metals from such sources [2–8, 10–13, 29–32]. Speciation analysis, aiming to identify and quantify various chemical species of PGMs occurring under various conditions, and estimate their biological activity, are key tasks for the evaluation of environmental and human hazard. Examination of the metal content in physiological fluids and tissues, and the interaction of various complexes with biomolecules, are essential for evaluation of human hazard both from metals of environmental origin and those introduced into the human body through chemotherapy. Data on the distribution and metabolism of drugs in the human body are fundamental for explanations of the therapeutic effectiveness and side effects of metal complexes used as drugs.

This chapter describes analytical procedures of the highest potential for analysis of biological (physiological fluids and tissues) and environmental (plants, airborne particulates and dusts, soils and sediments) samples for Pt and Ru used in chemotherapy and for Pt, Pd, and Rh used as components of autocatalysts.

13.2 Sample Preparation

The quality of analytical results obtained in the analysis of biological and environmental materials substantially depends on the sample preparation procedures applied, particularly when low concentrations of the metals and species of low stability are determined in complex matrices. Concentrations below the detection limits (DLs) of the available instrumental techniques and interfering effects from the matrix components require separation and preconcentration procedures. Instrumental techniques applicable to direct examination of solid materials are valuable for minimizing the effect of sample preparation steps on the quality of obtained results. In the analysis of noble metal samples, in particular those of complex environmental origin, use of such techniques is strongly limited because of interference from matrix components and the heterogeneity of examined materials.

The availability of a representative sample is essential in the examination of environmental materials because of the tendency of the noble metals to heterogeneous distribution. Larger samples taken for analysis are advantageous for minimizing the effect of nonhomogeneity of the examined materials. Quantitative digestion of the samples and transformation of the analytes into species suitable for subsequent steps (separation, preconcentration, and detection) require special attention as a result of the high inertness of the metals to chemical attack, complexity of matrices, and difficulties with conversion of the metals into stable, strictly defined complexes under the conditions used. Sampling and storage conditions ensuring stability of the species of interest require attention. Special precautions are needed to avoid contamination from chemical reagents and vessels. Minimizing the amount of chemicals introduced into the examined samples, as well as the number of sample preparation steps, can be advantageous. Loss of noble metals is possible as a result of sorption on the vessel walls and the high tendency of their complexes for hydrolysis, particularly under weakly acidic and neutral conditions. The kinetics of sorption are affected by the vessel material and depend on analyte concentration. Quartz vessels are considered the most suitable for digestion and storage of samples because of lower sorption compared with other materials (e.g., Teflon). Careful cleaning of vessels throughout all analytical procedures is essential for reducing the blank values. Detailed reviews of sample preparation methods (sampling, digestion, separation, and preconcentration) used in analysis of noble metal content in various materials are available [33–37].

13.2.1 Clinical Samples

Sampling and storage conditions ensuring stability of the complex of interest in clinical material are of great importance for the identification, quantification, and recognition of biological activity. A close correlation between physiological and analytical conditions is fundamental. Biological stress generated under sampling and storage can affect the natural composition of the examined sample and substantially reduce its representativeness. Any sample treatment requires deep knowledge of the behavior of the examined complexes under the applied conditions, and validation of parameters affecting their nature and concentration [38]. This is also important for calibration procedures, particularly for examination of complexes of low stability. Storage and incubation conditions should be appropriate for maintaining the examined complexes in their pharmacologically active forms. Matching the composition of calibration solutions to real, biological matrices is important, particularly when biologically active forms are the products of biotransformation processes occurring under physiological conditions, for example, hydrated species of cisplatin, $PtCl(NH_3)_2(H_2O)^+$ [39], and Ru(III) complex HIn *trans*-$[RuCl_4(Ind)_2]$ (KP1019) [40]. Special precautions are required to avoid sample contamination from the devices and chemicals used.

The examination of blood samples directly after sampling and centrifugation of erythrocytes is recommended [41, 42]. Ultrafiltrates should be analyzed directly after sampling. Storage at low temperatures of ≤ 0 °C (blood), < -10 °C (ultrafiltrates), and -20 °C (-80 °C) (urine) for a limited time prior to detection is possible [43–45]. Dilution or digestion generally precedes detection of metals in clinical samples. Dilution of physiological fluids can be sufficient for decreasing the concentration of solids interfering with detection. Water, dilute HCl, dilute HNO_3, dilute solutions of NaCl and HCl, Triton X-100 in mixtures with, for example, 0.2 % HNO_3, 10 % HCl, EDTA, or water, are used as dilution media. The kind of medium used depends on the matrix and the detection technique. Extensive dilution of native samples can lead to loss in sensitivity. Precipitation of proteins under sample dilution can cause problems. Digestion of physiological fluids is generally a better approach when determining the total metal content. Digestion of organic matrix always precedes the determination of metals in tissue samples. Concentrated HNO_3, aqua regia, mixtures of $HNO_3 + HClO_4$, $HNO_3 + H_2O_2$, and $HNO_3 + H_2O_2$ plus aqua regia are often used as digestion agents. Ultraviolet (UV) photolysis is attractive for improving the effectiveness of digestion procedures. The advantages of UV photolysis were demonstrated by analysis of blood [46] and urine [47] of unexposed people for physiological Pd, Pt (Ir and Au), and Pd and Pt using the inductively coupled plasma mass spectrometry (ICP MS) technique. Lower amounts of chemicals were used compared with classical mineral acid digestion, resulting in lower procedural blanks. Urine samples were completely digested under UV photolysis only in the presence of H_2O_2 [47].

13.2.2 Environmental Samples

The complex nature of environmental samples, the need to determine ultratraces of noble metals, and the large amounts of matrix components interfering with detection in most instrumental analytical techniques require digestion and separation procedures capable of quantitative recovery of metals from the examined samples. Appropriate coupling of sample preparation procedure and detection method is a key problem in obtaining reliable results. Wet acid treatment, fire assay (FA), chlorination, and oxidizing fusion are widely used for the decomposition of environmental samples. Preliminary drying, crushing, and grinding (powdering) of the examined materials might be necessary.

Treatment with wet mineral acids (aqua regia; mixtures of HNO_3, HCl and HF; and mixtures of HCl and $HClO_4$) and microwave-assisted high pressure conditions are of high potential for effective digestion of various environmental materials (plants, airborne particulate matters, dusts, soils, and sediments) prior to detection of total metal content. The use of HF may be necessary in the case of silicate-based matrices to liberate noble metals occluded in silicate grains [48–51], although good agreement of results has been obtained for analysis of complex samples (e.g., street dust) digested under aqua regia treatment with and without HF [52, 53]. Two steps

of aqua regia treatment (aqua regia, HF/$HClO_4$/aqua regia) was found effective for digestion of highly complex airborne particulate matter [54]. Quantitative recovery of Rh from dust samples can be problematic using wet chemical treatment [55]. Digestion with HF can result in higher amounts of the matrix elements being converted into soluble species and an increase in their interference with PGM detection. Preliminary treatment of the examined samples with dilute HCl can minimize the amount of some non-noble metals in solutions obtained after complete sample digestion. The purity of acids used is important for avoiding of high blank values.

Fire assay is an attractive technique for determining extremely low concentrations of PGMs in complex and heterogeneous materials (e.g., soils and sediments) [56–62]. The technique can be used for the examination of large (up to 5–100 g) samples, which minimizes the effect of heterogeneous distribution of the metals. Preconcentration of noble metals in a small bead of the collector is an advantage of the FA procedure. The nickel sulfide collector is effective for the recovery of all noble metals from various samples. The analytical procedure uses fusion of the sample with powdered Ni, S, $Na_2B_4O_7$, Na_2CO_3, and SiO_2 at ca. 1000 °C. Noble metals are collected in the small NiS bead formed under fusion. Treatment of the bead with HCl eliminates most common elements. The bead is subsequently treated with a mixture of mineral acids (HNO_3 + HCl) for dissolution of the noble metals. Large amounts of chemicals introduced into the sample can cause problems with the detection of metals in the obtained solution and give high procedural blanks.

Chlorination using chlorine attack can be an attractive method for determination of noble metals in samples of complex composition. All noble metals undergo chlorine attack. Binary chlorides or salts dissolved in diluted mineral acids are the products of chlorination. Dry chlorination by chlorine passing over the sample, either alone or mixed with a small amount of NaCl, in an open tube at 500–600 °C, is the most promising method because of the low amounts of chemicals introduced into the sample, low blanks, and the possibility of direct combination with the detection technique. The effectiveness of such a digestion procedure compared with FA in the analysis of rocks was reported [63].

Alkaline oxidizing fusion is an effective way of dissolution of metallic powders, particularly of metals resistant to direct wet acid treatment (Ru, Os, and Ir), but is rarely used for decomposition of complex noble metal samples because of low recoveries (e.g., 34–84 % Pt, Pd, and Au from silicate materials) [64]. Low stability of the complexes formed under dissolution (water, HCl) of the melt and difficulties with quantitative conversion of analytes into complexes of strictly defined composition (suitable for subsequent separation) limit the applicability of the alkaline fusion method. Hydroxocomplexes easily formed in the solutions can cause problems with quantitative separation and preconcentration of the metals, particularly when using ion-exchange chromatography.

Separation and preconcentration of PGMs prior to detection are generally required in the analysis of complex environmental samples because of the low concentrations to be determined and large excess of matrix elements interfering with detection [34–36, 65, 66]. Liquid chromatography (LC) using ion-exchange

and chelating resins, sorption/solid phase extraction (SPE), and co-precipitation with a suitable collector are the most often used techniques for such purposes. Ion-exchange chromatography, using both cationic and anionic exchangers, enables easy separation of the noble metals in solution (in the form of stable anionic complexes) from cationic forms of common elements. Anionic chloride complexes of the noble metals are retained on anionic resins, while base metals existing as cations under the conditions used pass through the resin. Complexes of the noble metals quantitatively pass into the eluent if using cationic columns, retaining positively charged species of non-noble elements. Quantitative conversion of PGMs into the appropriate anionic complexes, and non-noble metals into cations, is fundamental for their effective separation. Common metals that can exist in the examined solutions in the form of anionic species can behave similarly to anionic complexes of the noble metals in the chromatographic procedure used. An example is hafnium, which is prone to the formation of anionic fluoride complexes in the presence of hydrofluoric acid used for sample digestion [67]. The interfering effect of hafnium on the detection of platinum by ICP MS is a source of error [68–70]. Preliminary chelation of hafnium with morin allowed its separation from platinum on widely used Dowex 50W-X8 cationic resin as compared with pure chloride media [71]. Yttrium and zirconium anionic species cause difficulties with effective separation of palladium by ion-exchange procedures [68]. Strong affinity of anionic complexes of noble metals for anionic resins makes their quantitative recovery difficult. The use of relatively aggressive conditions (e.g., 12 mol/L HNO_3 at 83 °C) can be helpful for the elution of Pt (>95 % recovery) [72].

Chelating sorbents containing functional groups capable of forming stable complexes with the noble metals present in the examined solutions can be more efficient for their separation and preconcentration than ion-exchange resins. The noble metals easily form stable complexes with ligands containing sulfur (e.g., dithizone, dithiocarbamate, thiocarbocarbazide, 2-mercaptobenzothiazole, and thiourea derivatives) and nitrogen (e.g., amines, amides, and heterocyclic nitrogenous compounds). The determination of metals in the sorbent after dry ashing and wet acid treatment at high temperature and pressure, or directly in the solid phase by X-ray fluorescence, is promising, particularly when quantitative elution of the metals from the sorbent phase is difficult. Platinum and palladium were determined in soil samples after sorption on dithizone sorbent and elution with thiourea and concentrated HNO_3 (98 % Pt and 95 % Pd recoveries) [73]. Enrichment factors of 14 [74] and 41.7 [75] of platinum from soil samples were reported after sorption on Dowex 1X8 resin and silica gel, respectively, and immobilization with thiocarbohydrazide derivatives. Complexes of Pt, Pd, and Rh with rhodanine derivatives (e.g., 4-carboxyphenyl-thiorhodanine) [76] were found applicable for column separation and enrichment. Chelating sorbents can be useful in the analysis of complex samples containing high amounts of matrix elements interfering with detection and not completely separated by a single (e.g., ion chromatographic) step. The use of two-dimensional chromatography (cationic AG 50W-X8 and C18 immobilized with *N*,*N*-diethyl-*N*′-benzylothiourea resins) substantially improved the effectiveness of separation of Pd from highly abundant elements (e.g., Sr, Rb, and Y) that

interfere with its detection by ICP MS [77]. Large amounts of base metals in the examined samples cannot be quantitatively retained using only cationic resins.

Carbon-based sorbents are relatively new materials for the analysis of noble metal samples of different origin [78–84]. The separation and enrichment of palladium from water, fly ash, and road dust samples on oxidized carbon nanotubes (preconcentration factor of 165) [83]; palladium from road dust samples on dithiocarbamate-coated fullerene C_{60} (sorption efficiency of 99.2 %) [78], and rhodium on multiwalled carbon nanotubes modified with polyacrylonitrile (preconcentration factor of 120) [80] are examples of the application of various carbon-based sorbents for extraction of noble metals from environmental samples. Sorption of Au(III) and Pd(II) on hybrid material of multiwalled carbon nanotubes grafted with polypropylene amine dendrimers prior to their determination in food and environmental samples has recently been described [84]. Recent application of ion-imprinted polymers using various chelate complexes for SPE of noble metals such as Pt [85] and Pd [86] from environmental samples can be mentioned. Hydrophobic noble metal complexes undergo separation by extraction under cloud point extraction systems, for example, extraction of Pt, Pd, and Au with *N*, *N*-dihexyl-*N*′-benzylthiourea-Triton X-114 from sea water and dust samples [87].

Reductive co-precipitation of noble metals with a suitable collector (Te, Se, As, Hg, and Cu) may provide uncertainty when examining complex environmental samples as a result of difficulties with complete separation of high amounts of common metals, which can partially pass into the precipitate, and possible loss of PGMs. Recovery of Pt from soil samples was reported to be 55–87 % after its separation by co-precipitation with Te [88]. Long analysis time is a disadvantage of such a procedure.

High preconcentration factors in the analysis of environmental samples were obtained using electrolysis of the examined solutions and electrodeposition of noble metals (e.g., Pd and Pt in airborne particulates [89] and Pt, Pd, Rh, and Ru in road dust [90]) on the inner surface of pyrolytically coated graphite tube.

13.3 Instrumental Techniques for the Detection of PGMs

Detection techniques of high sensitivity, selectivity, and ease of coupling with sample preparation procedures are of special interest for measuring PGM content in biological and environmental samples. ICP MS, electrothermal atomic absorption spectrometry (ET AAS), adsorptive voltammetry (AV), and neutron activation analysis (NAA) have found the widest applications, both for direct determination of the total metal content in the examined samples and for coupling with instrumental separation techniques. Mass spectrometry coupled with techniques such as electrospray ionization (ESI) and capillary electrophoresis (CE) (e.g., ESI MS^n, LC ESI MS^n, LC ICP MS, CE MS^n, and CE ICP MS) offer powerful potential for speciation analysis of metals. MS is widely used for examination of the distribution of the metals in various materials (elemental analysis) and for elucidation of the

Table 13.1 Spectral (isobaric and polyatomic) interferences in the detection of Rh, Pd, and Pt by ICP-MS

Isotope	Abundance (%)	Interference
^{103}Rh	100	^{206}Pb, $^{86}Sr^{16}O^{1}H$, $^{87}Sr^{16}O$, $^{63}Cu^{40}Ar$, $^{87}Rb^{16}O$
^{105}Pd	22.2	$^{88}Sr^{16}O^{1}H$, $^{89}Y^{16}O$, $^{65}Cu^{40}Ar$
^{106}Pd	27.3	^{106}Cd, $^{90}Zr^{16}O$, $^{89}Y^{1}H$
^{108}Pd	26.7	^{108}Cd, $^{92}Zr^{16}O$, $^{92}Mo^{16}O$
^{110}Pd	11.8	^{110}Cd, $^{94}Zr^{16}O$, $^{94}Mo^{16}O$
^{194}Pt	32.9	$^{178}Hf^{16}O$
^{195}Pt	33.8	$^{179}Hf^{16}O$
^{196}Pt	25.3	^{196}Hg, $^{180}Hf^{16}O$, $^{180}W^{16}O$, $^{180}Ta^{16}O$

molecular structure of metal complexes. Molecular data are of great value for investigation of the interaction of various metal complexes with biomolecules and are the basis for evaluation of their biological activities.

ICP MS offers attractive detection limits of parts per billion (ppb) and sub-ppb plus selectivity, multielement capability, unique ability to measure isotopic ratios, wide dynamic range, and high speed of operation. It is therefore the preferred detection technique for elemental analysis of various materials. The identification and quantification of specific elements (e.g., heteroatoms) allows investigation of organic compounds undergoing destruction under plasma conditions, which can help in examination of the interactions of metals with biomolecules. Direct use of the technique for quantification of PGMs in complex samples, particularly in some environmental samples, can be strongly limited by interfering effects from the matrix components (Table 13.1) [48, 91–93]. Hafnium oxide (HfO^+) generated under plasma conditions seriously affects the ICP MS signals of all platinum isotopes. Polyatomic species of copper ($ArCu^+$), yttrium (YO^+), strontium (SrO^+), and zirconium (ZrO^+) are the source of spectral interference in the detection of palladium. The main interference with rhodium detection comes from polyatomic species of copper ($ArCu^+$) and rubidium (RbO^+), and from Pb^{2+} ions. Reliable results require identification, quantification, and elimination of the interference prior to the detection of PGMs in unknown samples. The content of the interfering species in the examined solution, the type of mass spectrometer used, and measurement conditions all affect the intensity of the interference. Double-focusing sector field mass spectrometers offering substantially better resolution (0.001 Da) than widely used quadrupole filters (1 Da) and are the better choice when determining Pt, Pd, and Rh in complex samples such as airborne particulate matter, dust, soil, and sediment [53, 94, 95]. Modifications in sample introduction techniques to obtain "dry" plasma conditions, under which the intensity of molecular ions containing O^- and OH^- are substantially reduced compared with those obtained in wet plasma, can help overcome the interferences occurring in the determination of metals in complex matrices. Electrothermal vaporization, thermospray nebulization, laser ablation, and membrane desolvation of sample aerosol are applied for such purposes. Mathematical correction is generally efficient for the elimination of

remaining interference. Dynamic reaction cells allow conversion of interfering ions into inactive species and can substantially reduce the interfering signals (e.g., Hf and Zr in the detection of Pt and Pd) and improve the DLs of the analytes [96, 97]. Spectral interference in the detection of noble metals can be efficiently overcome by introduction of a preliminary chemical separation step. Chemical separation is advantageous in the analysis of environmental materials such as soils and sediments that contain a large excess of interfering components. Simultaneous preconcentration of PGMs is attractive while determining their extremely low concentrations. Sample preparation steps can substantially affect selectivity and sensitivity of ICP MS results. High salt concentrations in the examined solution can lead to suppression or enhancement of the registered analyte signals. Special attention should be paid to the purity of the reagents, the vessels used, and the instrument analytical path to avoid contamination and possible memory effects, which can cause serious errors in the results obtained when quantifying traces of metals in the examined solutions.

Atomic absorption spectrometry (AAS) is applicable for the determination of PGMs both in clinical and environmental materials. Graphite furnace AAS provides better DLs than flame AAS and can be directly applied for the examination of materials of relatively simple matrices such as clinical samples, in particular those after chemotherapy treatment that contain large amounts of metals. Sample dilution and centrifugation (physiological fluids) might be sufficient for reducing matrix interference (cell constituents, proteins, salts, and lipids). Digestion, separation of metals from matrix components, and preconcentration often precede the detection of noble metals in more complex environmental materials. SPE and co-precipitation with a suitable collector are preferable for preconcentration of metals. Mercury is an attractive PGM collector as a result of its easy volatilization and lack of interference effect when using ET AAS detection.

Detection limits comparable with, or sometimes even better than, those offered by ICP MS can be achieved by the use of adsorptive voltammetry (AV) [98]. The technique was found applicable to the determination of physiological levels of Pt in blood ($\leq$0.8–6.9 ng/L) and urine (0.5–15 ng/L) samples [99]. The detection of rhodium by AV with 100-fold better DL (33 ng/kg) than obtained using ICP MS (2500 ng/kg) was reported [100]. Complexes of Pt(II) with formazone and Pd with dimethylglyoxime accumulated on the surface of a hanging mercury drop electrode, indicating the basis of most voltammetric procedures for both metals. Close reduction peak potentials and severe matrix effects limit direct application of AV for simultaneous detection of PGMs. Optimizing the electrolyte composition can allow simultaneous detection of Pt, Pd, and Rh [101] and of Pt and Rh [102]. AV is extremely sensitive to organic matrices. Limiting carbon content (to $\leq$0.1 %) by destroying the organic matrix is recommended prior to detection. Separation and preconcentration steps used prior to AV detection of PGMs in various matrices have been described [103].

Neutron activation analysis (NAA) is based on the production and detection of specific element radionuclides and is an attractive technique because of high sensitivity of detection and a practical lack of blanks [104]. Platinum, palladium,

and rhodium are determined by thermal neutron activation of the isotopes ^{198}Pt (n,γ)^{199}Pt → ^{199}Au ($t_{1/2} = 3.15$ days), ^{108}Pd(n,γ)^{109}Pd ($t_{1/2} = 13.5$ h), and ^{104}Rh ($t_{1/2} = 42$ s) [105–107]. The technique requires access to an irradiation source for producing specific radionuclides. Direct examination of complex environmental samples containing extremely low PGM concentrations can be problematic because of interference from matrix elements, particularly when long irradiation time and high neutron flux density are required to achieve sufficient sensitivity. Suitable radiochemical procedures for separation of metals from the examined samples are often applied in the analysis of both biological and environmental materials. The combination of FA with NAA is attractive for the examination of complex samples (e.g., soils and sediments). Nuclear analytical techniques have also found applications in speciation analysis of biological and environmental samples [108].

13.4 Platinum and Ruthenium in Clinical Samples

In 1978, cisplatin (Fig. 13.1) was introduced into chemotherapy as a particularly effective drug against lung, ovarian, bladder, head, and neck cancers. Limitations in the application of cisplatin, because of severe side effects and development of drug resistance of some tumors during clinical treatment, have stimulated intensive worldwide studies of second generation compounds with better antitumor characteristics and safety profiles. From a huge number of investigated compounds, carboplatin and oxaliplatin have been introduced into clinical use worldwide (in 1992 and 2003, respectively); some drugs (lobaplatin, nedaplatin and heptaplatin) have been locally (China, Japan and South Korea, respectively) approved. Numerous (ca. 40 up to 2008 [21]) compounds have been put through clinical trials, for example, satraplatin (JM-216) and ZD0473 (Fig. 13.1). Detailed descriptions of studies of the therapeutic activity of classical and newly designed Pt (II)- and Pt(IV)-based compounds of different composition, complexing ligands, and structures are available [14, 17, 19–21, 109, 110].

Ruthenium complexes active against some tumors, in particular those resistant to cisplatin, have recently attracted attention. They are considered the most likely candidates for the next generation of anticancer drugs. Among numerous investigated ruthenium compounds with various ligands (e.g., amine, dimethylsulfoxide, polyaminepolycarboxylate, heterocyclic), complexes with imidazolium (Im) and indazolium (In) were found to be the most promising and selected for clinical development. Examples include [HIm][*trans*-[$RuCl_4(Im)_2$] (KP418), [HIn][*trans*-$RuCl_4(In)_2$] (KP1019), [HIm][*trans*-$RuCl_4$(DMSO)Im] (NAMI-A), and Na[*trans*-$RuCl_4(In)_2$] (KP1339)] (Fig. 13.2). The lower side effects of Ru-based complexes compared with cisplatin and their ability to inhibit metastatic progression have stimulated intensive research on such compounds [14–16, 18, 22, 25, 111–113].

Current challenges in clinical and pharmaceutical studies are to explain the mechanism of cytotoxic activity of drugs, their transport into cancer cells, distribution and metabolism in the human body, interaction with proteins and with DNA

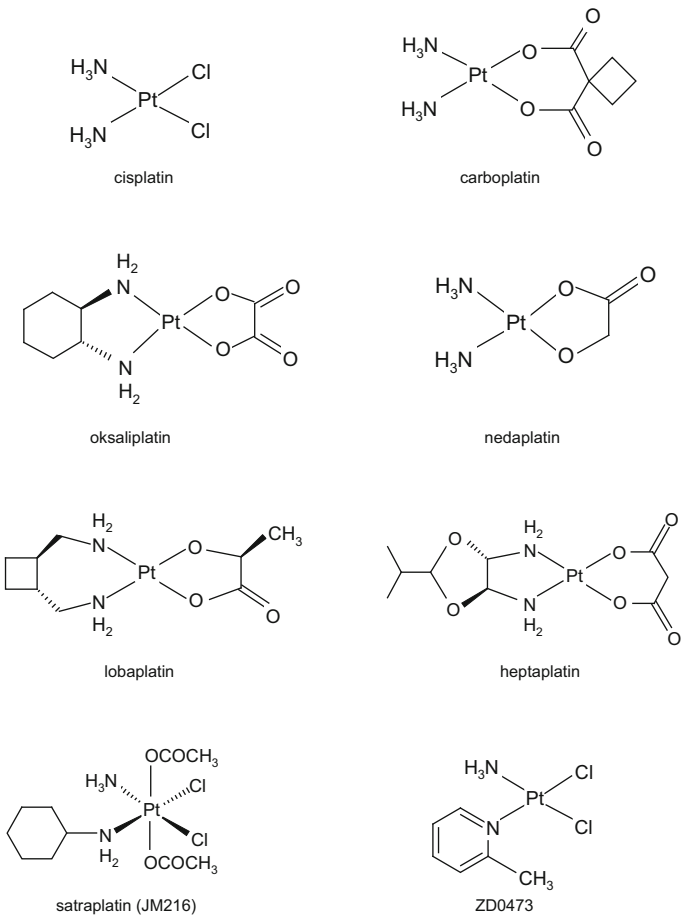

Fig. 13.1 Cisplatin and some second generation compounds with anticancer properties

(being the target drug molecules), and side effects [114–118]. Analytical results on the element content in physiological fluids and tissues provide data on the distribution of administered drug in the human body. Speciation analysis is of great value in explaining the interaction of drugs with target biological molecules. In-vitro experiments involving incubation of the complexes with biological ligands that occur under physiological conditions play an important role in the investigation of their mechanism of action. Such simulation experiments require the composition of the in-vitro examined matrix to be as close as possible to that of the biological material of the interest.

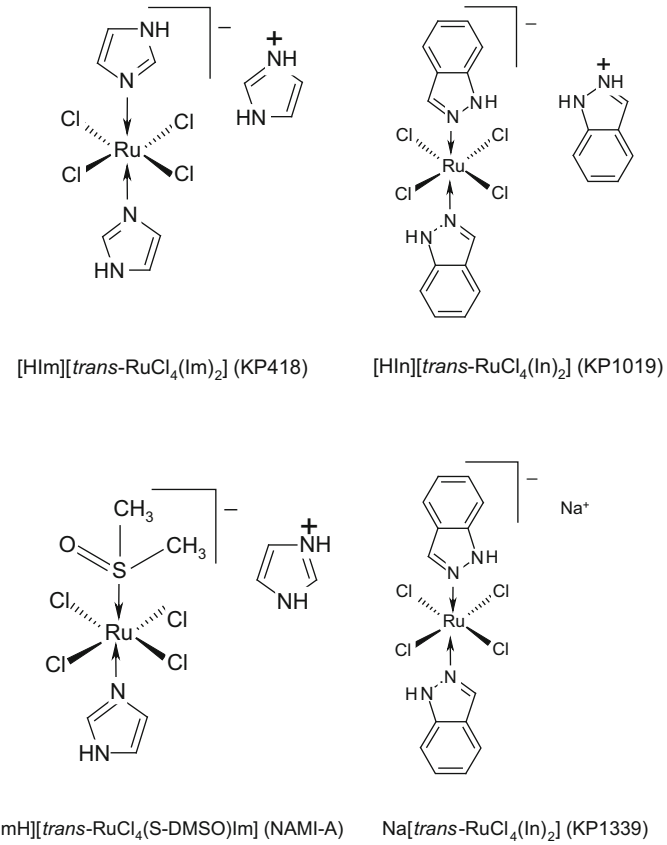

Fig. 13.2 Examples of clinically investigated ruthenium anticancer compounds

13.4.1 Determination of Platinum in Clinical Samples

The determination of the total platinum content in physiological fluids and tissues, both during clinical treatment or after environmental exposure, requires instrumental techniques of sufficient DLs and selectivities. ICP MS provides the most attractive DLs for platinum in biological samples, for example, 7.50 ng/L in human plasma ultrafiltrate after chemotherapy with cisplatin, carboplatin, and oxaliplatin [119]; 0.1 μg/mL in blood, serum, and ultrafiltrate samples after chemotherapy with oxaliplatin and 5-fluorouracyl [120]; 26 pg/g in DNA isolated from cancer ovarian cells after different exposure times and concentrations of cisplatin [121]; and 0.75 pg in DNA extracts from peripheral blood mononuclear cells and tissues from patients treated with cisplatin [122] and 1.0 μg/L in serum, 0.1 μg/L in ultrafiltrate, and 2 μg/L in urine [123]. The ICP MS technique allowed detection of physiological levels of Pt in the unexposed human body: 0.3–1.3 ng/L in blood (DL of 0.3 ng/L) [46]; 0.48–7.7 ng/L in urine (DL of 0.24 ng/L) [47]; and 0.778 ng/g

in lung, 0.031–1.42 ng/g in liver, and 0.051–0.422 ng/g in kidney tissues (DLs of 20, 20, and 34 pg/g of dry weight, respectively) [124]. Total content of platinum in physiological fluids and tissues can also be determined by ET AAS [44, 125–135], AV [99, 136–138], and NAA [139–141]. AAS was found applicable for determination of Pt in various peptide fractions (<5000, <50,000, and <100,000 Da) isolated by ultrafiltration from samples with high protein content [142], in DNA complexes after separation by CE [143], and for examination of the decomposition products of carboplatin (by high-performance liquid chromatography, HPLC) during long-term storage (5–78 months) [133]. Because of the extremely low DLs achieved, AV was found applicable for examination of long-term levels (over 10 years) of Pt in patients treated with Pt-based drugs [136, 137]. Platinum in human and animal tissues was determined by NAA after electrolytic separation of gold on a niobium cathode [141], extraction of Au-diethyldithiocarbamate complex [140], precipitation of Au, and extraction of Mo (which can accumulate in cancer cells) [139]. The bioaccumulation coefficient of cisplatin in cancer cells was reported to be 1.8–3.8.

Separation techniques, in particular ion chromatography and CE, coupled with element-specific and molecular detectors enable investigation of various platinum species occurring under physiological conditions, which is fundamental for explanation of the metabolism and therapeutic properties of these drugs [42, 116, 144–152]. The monohydrated complex of cisplatin, a product of hydrolysis occurring under low chloride concentrations in cancer cells, is considered the most reactive with DNA. A minor fraction of low molecular weight platinum species was identified by two complementary LC ICP MS techniques after 24 h incubation of proliferating living cells (mesothelioma cell model P31) with preclinically relevant cisplatin concentrations [153]. The determination of intact cisplatin and its monohydrated complex in human plasma [154] and of metabolites of drug ZD 0472 in urine [155] and animal plasma ultrafiltrate [156] are examples of the application of HPLC ICP MS and HPLC MS/MS techniques. Substantial improvement in DLs of cisplatin and its hydrated complex determined by LC ICP MS was achieved by using dimethylformamide as modifier of the mobile phase [157]. Substantially better (by a factor of 100) sensitivity and lower uncertainty of the quantification of carboplatin in patient urine samples were achieved by the use of HPLC ICP (Q)MS compared with HPLC ESI (TOF)MS [158].

Investigation of the interaction of platinum drugs with blood proteins, albumin, and transferrin [159]; oxaliplatin with hemoglobin in blood samples of colorectal cancer patients [160]; the adducts of ZD 0743 with urine proteins [161]; and cisplatin, transplatin, and oxaliplatin with low molecular weight proteins [162] are examples of the potential of HPLC ICP MS, ESI MS, MALDI MS, and tandem MS techniques in such studies. Interaction of the drugs with sulfur-containing ligands (methionine and cysteine) can result in the generation of adducts of higher toxicity than the parent compounds. The examination of such adducts by evaluation of the Pt:S ratio by ICP MS is useful [163]. The reported DLs for cisplatin, *cis*-[$PtCl(NH_3)_2(H_2O)]^+$, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, and four adducts with methionine were, respectively, 0.31, 0.25, 3.83, 1.07, 0.56, 0.82, and 2.38 μg/L. Liquid

chromatography coupled both with elemental and molecular MS detectors is valuable for investigation of the interaction of the drugs with DNA in living cells [164–166]. CE offers the possibility of investigating the interaction under conditions corresponding to physiological conditions (aqueous media, pH, and temperature) and is valuable for such studies [146, 149, 167–172]. Gel electrophoresis coupled with ICP MS and MALDI TOF MS has proved useful for examination of the interaction of cisplatin with oligonucleotides [173].

13.4.2 Determination of Ruthenium in Clinical Samples

The oxidation state of ruthenium and the type of coordinated ligands and biotransformation processes occurring under physiological conditions affect the pharmacological activity of complexes examined as potential anticancer drugs. Ruthenium can exist under physiological conditions as Ru(II), Ru(III), and Ru(IV). Complexes of Ru (II) and Ru(III) attract most attention, Ru(III) because it is more biologically inert than Ru(II) and Ru(IV), and Ru(II) because it exhibits the highest biological activity. Ru(II) species are the products of reduction of Ru(III) in cancer cells. Low pH (5–6) and lower molecular oxygen concentration in cancer cells, as compared with healthy conditions, and the presence of reducing agents (ascorbic acid, glutathione) stimulate the reduction of Ru(III) to Ru(II). The administration of cytostatic complexes of Ru(III), which are reduced to Ru(II) in cancer cells, is favorable for reducing the toxicity of drugs. Complexes of Ru(III), KP1019 and its Na^+ analogue KP1339, NAMI-A, and organometallic complexes of Ru(II) with RAPTA ligands $[Ru(\eta^6\text{-}C_6H_5Me)(PTA)Cl_2]$ [174–177], are the most prominent antimetastatic complexes, with better anticancer activity and selectivity than platinum complexes.

Analytical studies of the pharmacological activity of various complexes are focused on examination of the distribution of ruthenium in the human body (physiological fluids and tissues), mechanisms of transport into cancer cells, interactions with plasma proteins, hydrolysis and redox reactions, and the interaction with DNA. Substantial effort has been undertaken to explain the interaction of complexes with transferrin, which plays a dominant role in their transport into cancer cells. Cancer cells have a higher (2–12 times) number of transferring receptors than healthy cells [174]. The synergetic role of bicarbonate anions in binding of some ruthenium complexes by transferrin has been emphasized [178, 179]. Studies of the interaction of KP1019 complex have shown its substantially stronger binding with plasma proteins, albumin, and transferrin than with nucleotides. Most Ru is bound to albumin, the most abundant plasma protein. On the basis of results obtained by two-dimensional size-exclusion/anion-exchange chromatography coupled with ICP MS, less than 20 % of KP1019 complex was expected to bind to transferrin in human plasma in an equimolar mixture of albumin and transferrin, and <2 % in an incubation mixture containing tenfold excess of albumin (5×10^{-5} mol/L) to transferrin (5×10^{-6} mol/L), corresponding to physiological conditions [180]. Strong affinity of another Ru complex, NAMI-A, to

albumin (electrochemical studies) has also been recognized [181]. Various techniques were found useful for examination of the binding mechanism of pharmacologically active ruthenium complexes to serum proteins, such as spectrofluorimetry, ultrafiltration-UV-vis spectrophotometry and CE (for binding of KP1039 and KP1339 to albumin) [182], circular dichroism spectroscopy and ESI MS (for binding of KP1019 to transferrin) [179], CE with UV [183] and ICP MS [184] detectors, and electron paramagnetic resonance [185]. Large protein complexes/aggregates (above 700 kDa) as initial major binding partners (KP1019 and KP1339) in cytosol, followed by ruthenium redistribution to the soluble protein fraction (below 40 kDa), were identified by tandem size-exclusion chromatography (SEC/SEC) in combination with ICP MS [186]. CE and ESI MS/MS techniques are particularly useful for studies of the mechanisms of biological interaction of Ru-based complexes [111, 187, 188]. Correlation of in vitro results with those obtained under physiological conditions can be problematic as a result of insufficient DLs of the analytical procedures and matrices that do not strictly correspond to each other. Direct (after dilution or digestion) examination of clinical samples by ICP MS or ET AAS is the most often used method for evaluating the total content of ruthenium.

13.5 Quantification of Pt, Pd, and Rh in Environmental Materials

The problems of the presence of PGMs in various environmental compartments and human exposure to them have been discussed in numerous review articles and books. [2–8, 10–13, 189]. We describe analytical approaches to the evaluation of the content of Pt, Pd, and Rh in plants, airborne particles and dusts, and soils and sediments, which are the most often examined materials for such purposes.

13.5.1 Plants

Plants have the potential for bioaccumulation of metals and their use as food can cause direct introduction of PGMs into the human diet. Plants therefore attract particular attention in analytical studies aiming at the evaluation of human exposure. The interest in examination of environmental plants for PGM content was stimulated by data on the substantially higher content of metals in plants such as spinach, cress, phacelia, and stinging nettle cultivated on soils close to highways (8.6 μg/kg Pt, 1.9 μg/kg Pd, and 1 μg/kg Rh) than on reference uncontaminated soils (amounts close to DLs of the metals) [190]. Results published shortly after the introduction of autocatalysts clearly indicated the bioaccumulation of metals in plants cultivated near highways. Palladium exhibited the highest bioaccumulation

potential in plants compared with Pt and Rh ($Pd > Pt \geq Rh$) and similar bioaccumulation as Cu and Zn, and in some cases Cd. The uptake of metals by plants occurs in the decreasing order: root, steam, leaves. Mosses are often used as biotest samples for evaluation of the environmental impact of PGMs. Data on the content of various metals in mosses has been published: 6.4–27.4 ng/g Pt and 1.2–4.6 ng/g Rh in one study [191] and 30 ng/g Pt, 2.4 ng/g Pd, and 5.4 ng/g Rh in another [192].

ICP MS and AV offer the potential for direct detection of metals in solutions obtained after sample digestion. Improvement in the DLs of such techniques can be obtained by suitable coupling of other detection techniques with sample preconcentration procedures, for example, AAS with electrodeposition of Pt into a graphite tube [193] or sample evaporation [194]. AV is attractive because of the adequate DLs and low instrumental and operating costs. It is often applied for the examination of plant materials. The determination of Pt (0.085 ± 0.004 μg/g) and Pd (0.096 ± 0.005 μg/g) in laurel leaves [195], Pt (0.157–0.240 μg/kg) in camomile [196], Pt (543 pg/g) and Rh (22 pg/g) in spruce shoots [197], and Pt (0.03–10 μg/kg) and Rh (0.03–21 μg/kg) in grass samples [100] are examples of analytical results obtained using AV. Determination was also made of platinum in grass, with values ranging from 19.1 ± 1.6 ng/g in leaves to 136 ± 2 ng/g in roots, by a voltammetric method using a solid electrode of glassy carbon [198].

Identification and quantification of metal–biomolecule species occurring in plants are challenging problems in elucidating their metabolic processes and human exposure [199]. Such data are still limited as a result of difficulties with the separation of native species from samples and insufficient DLs of the available instrumental techniques. Data has been published on the occurrence of metals in inorganic and organic species in plant materials, for example, 90 % Pt in inorganic and 10 % in organic forms in grass samples exposed to a solution of $Pt(NH_3)_4](NO_3)_2$ [200], and 23 % Pd in the peptide fraction of > 10 kDa (gel permeation chromatography and X-ray fluorescence) [201]. Over 90 % of Pt was identified in fractions of about 1 kDa, and the rest in 19 to >1000 kDa fractions (SEC and UV detection) [202].

13.5.2 Airborne Particles and Dusts

Evaluation of the content of PGMs in airborne particles and dusts is important because of the possibility of their inhalation and accumulation in human lungs. Nanoparticles from autocatalysts can be transported into various parts of the environment (waters, plants, soils, and sediments) and transformed into more bioavailable species. There are data on the higher solubility of platinum from tunnel dusts than from inorganic species emitted from converters [30]. Distribution and accumulation of metals depend on traffic density, distance from the road, and meteorological conditions (wind, rain). The age of an autocatalyst and speed conditions directly affect the amount of nanoparticles released from catalytic

converters. This can result in wide variations in metal content in samples taken from various areas, and low correspondence with metal content in the areas of the interest (e.g., high traffic zones in urban areas compared with suburban sites) [203, 204]. Substantial differences in metal content and distribution can be observed in airborne particles of various sizes [205, 206].

The complexity of airborne particles and dusts, difficulties with their complete digestion, and large excess of matrix elements interfering with the detection of PGMs by instrumental techniques are challenging analytical problems. The concentrations of Hf, Cu, and Pb exceed the concentrations of PGMs by several orders of magnitude, resulting in difficulties with the detection of PGMs by ICP MS [48, 49]. Preliminary treatment with HCl can result in a decrease in base metal content in the final examined solution, without PGM loss [93]. The application of mathematical correction [48, 205], dry plasma conditions obtained by modification of sample introduction into the plasma [53, 205, 207, 208], dynamic reaction cells [97], sector field instruments [53], and chemical separations [50, 209] are used for the elimination of interferences in the detection of the metals in airborne matters by ICP MS. Quantitative separation and preconcentration of Pt from dust samples (CRM BCR-723) by cloud point extraction prior to ICP MS detection has been reported [210].

Pretreatment steps resulting in separation and preconcentration of PGMs are generally required when using AAS for detection. Examples of such procedures are electrodeposition of Pt (82.8 μg/g), Pd (61.3 μg/g), Rh (19.6 μg/g), and Ru (<DL) on a graphite tube [90]; adsorption of Pt (20–34 pg/m^3) [211] and Pd (4–16 pg/m^3) [212] on a C18 microcolumn loaded with *N*,*N*-diethyl-*N*′-benzoylthiourea; preconcentration of Pd on fullerene C60 loaded with DDTC (179.2 ng/g) [78]; and preconcentration on silica gel as a complex with dimethylglyoxime (0.22–0.23 μg/g) [213]. A considerable increase in analytical features of techniques used in determination of Pt, Pd, and Rh in road dust (BCR-723) was achieved by appropriate combination of extraction conditions and atomization program [214]. Separation and preconcentration procedures generally precede detection of metals by NAA [215–217]. Ion-exchange chromatography using the anionic Dowex 1X8 column was applicable for determination of platinum in road dusts (14–16 ng/g) [216] and in CW7 (50.4 ± 2.0 μg/kg) and CW8 (76.8 ± 2.7 μg/kg) candidate reference materials [217]. Extremely low DLs were reported for Pt (0.5 pg/m^3 [218] and 0.5 ng/g [219]) in airborne particles and road dusts using AV. Different Pt contents have been reported, depending on sampling site (e.g., 3.0–33.0 pg/m^3 Pt [218] and 0.02–5.1 pg/m^3 Pt [220]).

13.5.3 Soils and Sediments

Soils and sediments can accumulate large amounts of PGMs. Palladium exhibits the highest affinity for such matrices, in particular those of high clay content and exchangeable metallic fractions [221]. Platinum generally dominates in such

samples as a consequence of its highest content in autocatalysts. Platinum concentrations in soils at roadsides (Frankfurt–Mannheim, Germany) were 70 times higher than the original geogenic background values [60]. The background values of Pt, Pd, and Rh (German areas) were estimated to be <1 μg/kg, ≤ 0.5 μg/kg, and <0.1 μg/kg, respectively [222]. The accumulation of metals in soils and sediments increases in areas near roads and decreases with increasing distance from the road, and with sampling depth. Physicochemical properties (pH, redox potential, salinity, and complexing agents) of soils and sediments can affect the conversion of metals into more soluble, mobile, and bioavailable species. Chemical oxidation, complexation, and biochemical transformation of metals through bacterial action results in their higher mobility.

The complex soil and sediment matrices and high excess of matrix elements require special attention when choosing an analytical procedure for the determination of traces of PGMs. Large interferences from matrix components substantially limit direct detection of metals in solutions obtained after sample digestion. The sector field (SF) ICP MS technique is of higher potential for the detection of PGMs in complex samples. It was found suitable for direct examination of solutions after FA digestion of sediments taken from an urban lake [62]. The determination of Pd in soils and sediments can cause difficulties, even using SF ICP MS [223]. There are data on the higher amounts of Pd determined in sediments by direct application of ICP MS technique (700 ng/g) than after its separation by extraction with diethyldithiocarbamate (21 ng/g) [192], or by laser ablation ICP MS (80 ng/g) compared with SF ICP MS (68 ng/g) after chemical sample digestion [224]. Difficulties with the elimination of interference from SrO^+ and RbO^+ in the detection of Rh by ICP MS cause problems with the reliability of the results. Chromatographic techniques or co-precipitation with Te are often applied for separation of analytes. Isotope dilution provides the possibility of quantification of metals if anionic resins are used and there are difficulties with complete elution of anionic PGM complexes. Dynamic reaction cells can improve the determination of metals. The elimination of Zr interference in Pd detection using oxygen as the reactant gas to convert Zr into oxides of a higher order is an example of dynamic reaction cell applicability [96].

13.6 Quality Control

The complex nature of noble metal samples (in particular those of environmental origin), the extremely low concentrations of the metals to be determined, and numerous interferences make the reliability of the obtained results a fundamental problem. The availability of adequate certified reference materials (CRMs) containing the analytes in strictly corresponding matrices and at similar concentrations is problematic. The available CRMs of geological matrices usually contain noble metals at microgram per gram concentrations [225–227], which limits their use for evaluation of the reliability of results in the analysis of environmental and biological samples containing nanogram per gram and picogram per gram levels. In

2001, CRM for road dust (BCR-723) containing 81.3 ± 2.5 μg/kg Pt, 6.1 ± 1.9 μg/kg Pd, and 12.8 ± 1.3 μg/kg Rh, was introduced [49, 228]. It is widely used for quality control of results obtained in the analysis of environmental materials (e.g., airborne particulate matters, dusts, soils, and sediments). Comparison of results obtained using different analytical procedures and interlaboratory studies are recommended when there is a lack of suitable CRM (e.g., in examination of clinical samples). The use of standards based on real matrices (e.g., saliva, plasma, ultrafiltrates, and lung fluids) instead of synthetic solutions is recommended in such analyses. Difficulties with the identification and quantification of different metal species in examined samples make the reliability of results of great importance. The use of various instrumental techniques for examination of particular samples can be helpful. The application of chromatography, mass spectrometry, and electrochemistry [199]; HPLC ICP MS and HPLC MS/MS [156]; ESI MS and MALDI [162]; micellar electrokinetic chromatography, NMR, and MS [167]; AAS, ESI MS, and CD spectroscopy [179]; SEC IC ICP MS and LC ESI MS [180]; and NMR and HPLC [229] are examples of such approaches.

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Chapter 14
Determination of Volatile Organic Compounds: Enrichment and Analysis

Bogusław Buszewski, Tomasz Ligor, and Agnieszka Ulanowska

14.1 Introduction

Modern analytical methods enable comprehensive and precise analysis of the whole range of compounds present in various matrices. For the sake of natural systems and their proper functioning, the determination of contaminants that irreversibly destroy living organisms is of utmost importance. Reducing emissions, or even halting production processes that result in release of harmful substances, is necessary. Because industry tends to be constantly expanding, both direct and indirect hazards cannot be avoided. Direct hazards involve the contamination of matrices such as water, soil, and atmosphere, whereas indirect hazards relate to food, plants, animals, and humans. To prevent the degradation of natural environments, regular control measurements are required to monitor changes occurring in ecosystems.

The development of modern analytical methods based on physicochemical phenomena and natural processes has led to lowering the detection limits and increasing the accuracy of measurement. The applicability of particular analytical techniques is related not only to the kinds and properties of substances detected but also to the selectivity and reproducibility of the method used for analyte isolation and enrichment (sample preparation stage). According to the literature, sample preparation constitutes the most important stage in qualitative and quantitative determination. Moreover, the time needed to complete sample preparation amounts to approximately 60–65 % of the total time required to accomplish the entire analytical process. Taking the whole variety of sample preparation methods into consideration, those based on surface phenomena (i.e., adsorption/desorption and/or extraction) taking place on phase boundaries (gas–liquid, gas–solid, liquid–liquid, or liquid–solid) are the dominant methods applied in routine trace analysis.

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This results from the fact that these techniques can be directly combined (in-line and/or off-line) with other physicochemical measurement methods such as gas chromatography (GC) or high-performance liquid chromatography (HPLC), allowing process automation. Consequently, high data reproducibility and high measurement accuracy, together with satisfactory cost-effectiveness for a single analysis, are obtained. In numerous variants of routine trace analysis it is the simplicity of determination, resulting from not only the operational process (using instruments) but also the analytical procedures, that is a significant advantage. Therefore, developing new methodology and analytical procedures as well as implementing them with the use of modern, high-quality equipment constitutes the fundamentals of modern analytics.

The choice of an appropriate measurement technique (e.g., chromatographic separation) depends on the physicochemical properties of the analyte (Fig. 14.1). According to the Giger definition, apart from molecular mass, chemical character, and polarity, the volatility of analytes must also be considered [1]. This factor determines the applicability of GC for the final determination of analytes of molecular masses up to 1000 Da and high vapor pressure.

Therefore, from the whole range of analytes, a significant group, the so-called volatile organic compounds (VOCs), can be distinguished. According to the US Environmental Protection Agency (EPA), vapor pressure above the value of 0.1013 kPa is the criterion for inclusion of a compound into the group of VOCs. Compounds that are gases under these conditions (e.g., methane), other hydrocarbons having up to four C atoms and their derivatives, aromatic molecules, and substances having more than ten C atoms in their structures are excluded from the group of VOCs.

Some VOCs are toxic and carcinogenic. Two main sources of VOCs can be distinguished: natural and anthropogenic. Natural sources are the vegetation processes of certain organisms, assimilation processes, forest fires, volcanic or geyser activity, and natural gas release (ca. 30–60 million tons per year). Anthropogenic

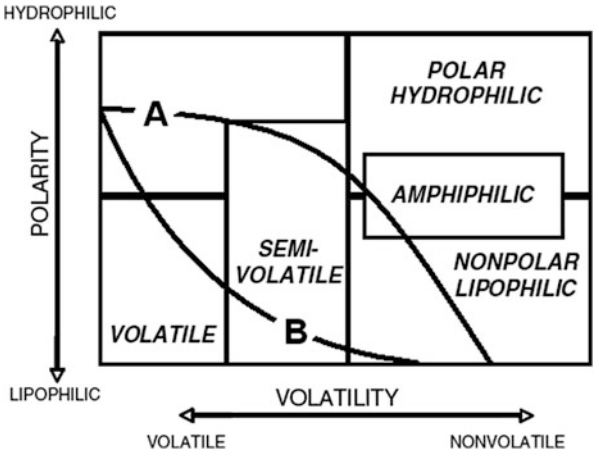

Fig. 14.1 Ranges of separation techniques applicable for analytes of varied physicochemical properties: *A* GC limits, *B* HPLC and CZE limits (according to W. Giger [1])

sources include fossil fuel exploitation and combustion processes, crude oil processing, metallurgy, organic chemical industry, solvent production and application, food processing industry, farming, solid waste management, and road, air, and marine transportation [2].

According to Pauling, of 10,000 VOCs 200 are emitted by living organisms and can be identified in the air exhaled by patients in the form of so-called specific odoriferous substances. These substances belong to groups such as alkanes, methylated alkanes, or aromatic compounds and their functionalized derivatives [3]. Thus, there has been growing interest in this topic in the world of medicine.

As can be seen from those simple examples, chemical monitoring of VOCs based on determination of their trace amounts (from parts per million [ppm] to parts per trillion [ppt]) with the use of modern analytical methods is necessary. It is a consequence of the ease with which VOCs permeate biological barriers (air–circulatory system, the internal and external activities of both plant and animal cells), and the relative ease with which they undergo conjugation reactions (enzymatic oxidation reactions, auto-oxidation, initiation, propagation, and termination). Therefore, new metabolites are created. These metabolites not only determine phenomena connected with the chemistry of the atmosphere but also are responsible for the vital functions of organisms (e.g., the uncontrolled de novo reaction that occurs in the presence of free radicals) (Fig. 14.2) [4].

Currently, the use of chromatographic techniques combined with spectral methods and properly chosen sample preparation methods seems to be the only

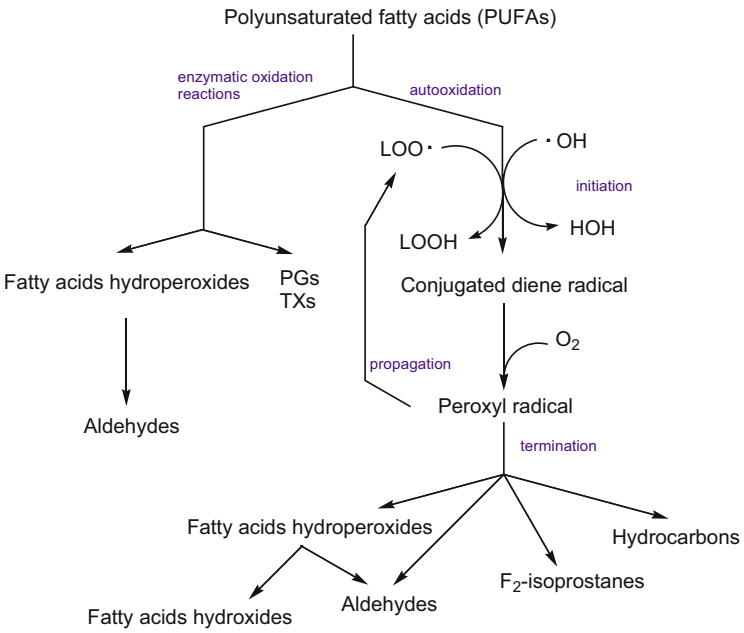

Fig. 14.2 Scheme of reactions occurring in the human body under oxidative stress [4]

option for fast, complex, relatively cheap (when calculating the unit cost of an analysis), and accurate determination of VOCs in various environmental and biological matrices.

14.2 Sample Preparation

Liquid–Liquid Microextraction The main advantage of liquid–liquid microextraction (μ-LLE) is that it is a simple application that does not require special equipment. A basic drawback of LLE is the need for flammable or toxic solvents. Many of the problems related to traditional LLE have been successfully solved.

In 1978, Karlberg and Thelander [5] described the flow injection extraction (FIE) technique, and in 1979 Murray [6] improved the microextraction, reducing the amount of solvent to 200 μL. The main disadvantage of these methods was the necessity to use complicated equipment. Jeannot and Cantwell [7] and, independently, Hee and Lee [8] introduced a simpler kind of microextraction in which a solvent drop is applied, single drop microextraction (SDME). They designed a microreactor with 8 μL of *n*-octane in a Teflon tube (Fig. 14.3) [7]. The authors performed measurements of diffusion coefficients and the kinetics of the system, which suggested a mass transfer model.

He and Lee [8] developed a method for microextraction in which an organic drop hangs on the tip of a GC syringe. This idea was presented in two modifications: dynamic and static. In the dynamic method, a conventional chromatographic syringe is used as a vessel for extraction. In the static method, a 1-μL drop of the organic solvent hangs on the tip of a needle. The extracts were analyzed by means of GC coupled with an electron capture detector (ECD). Buszewski et al. applied

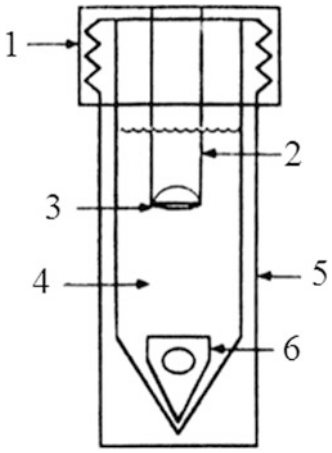

Fig. 14.3 Scheme of a microreactor: *1* cap, *2* Teflon rod, *3* organic phase, *4* aqueous phase, *5* vial, *6* stir bar [7]

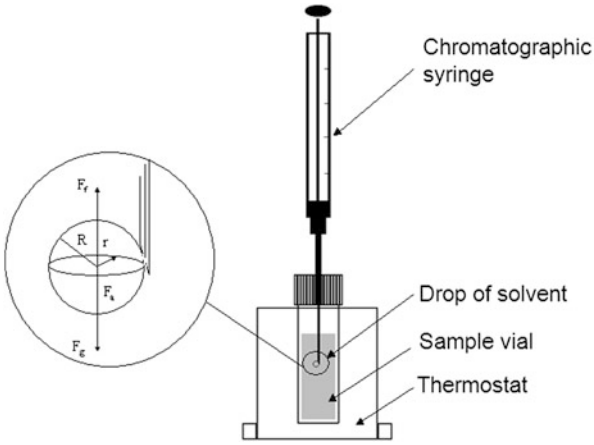

Fig. 14.4 Scheme for single-drop microextraction [10]

the static SDME method using a drop of hexane, and toluene for the extraction of chlorinated compounds [9]. The typical GC microsyringe with an angled tip needle has been used for this application, allowing stabilization of the hanging drop (Fig. 14.4) [10].

Calibration is carried out using standard calibration curves. The simplicity, repeatability, and low cost of the method have allowed its use for routine determination of trihalomethanes in tap water. SDME has also been compared with solid phase microextraction (SPME), purge and trap (P&T), and direct aqueous injection (DAI) [10]. This technique offers accuracy comparable with that obtained using P&T and DAI. With respect to conventional LLE, the SDME method is more accurate. In contrast to DAI and P&T, it requires no special equipment. SDME has been used for extraction of chlorophenols [11], pesticides [12, 13], warfare agents [14], and butanone derivatives [15], and for control of food products [16]. The low costs of the SDME method (typical GC syringe and 2–3 μL of solvent), simplicity, and short extraction time (approximately 15 min) make it particularly suitable for preliminary analyses of organic pollutants in water samples. It can also be an effective alternative to SPME, as it does not require the use of expensive instrumentation.

Dispersive Liquid–Liquid Microextraction The aforementioned SDME method, although it significantly reduces solvent consumption, is not free from drawbacks such as low extraction efficiency and slowly reached equilibrium. In many cases, the extraction efficiency can be increased by using dispersive systems such as the emulsion of organic solvent in an aqueous sample. In dispersive liquid–liquid microextraction (DLLME), a mixture of two solvents (extraction solvent and disperser) is injected by syringe into an aqueous sample. The extraction solvent is a water-insoluble and nonpolar liquid such as toluene, chloroform, dichloromethane, carbon tetrachloride, or carbon disulfide. A water-miscible, polar solvent, typically acetonitrile, acetone, isopropanol, or methanol, is used as disperser. The typical concentration of extractant in such a mixture is in the range 1–3 %.

A cloudy solution is immediately formed after rapid injection of mixture into the aqueous sample as a result of high dispersion of the organic phase in water (millions of fine droplets). Diffusion of analytes from the aqueous to the organic phase occurs much more effectively than in water. In order to separate the immiscible solvent, the emulsion is centrifuged and the organic phase deposited on the bottom of a conical vessel. Afterwards, the organic extract is injected into the chromatograph. This method is rapid, relatively simply to perform, proves high recovery, and gives high enrichment factors.

The DLLME technique was introduced by Rezaee et al. and applied for extraction of polycyclic aromatic hydrocarbons (PAHs), pesticides, and alkylbenzenes [17]; UV filters [18]; and chlorophenols [19] from water samples. Summarized applications can be found in review articles [20–24]. An interesting concept is the introduction of ionic liquids as extractants [25–28].

An improvement in the technique is the use of a solvent lighter than water. Hexane in a mixture of acetone (disperser) has been used for the extraction and determination of 16 PAHs. An important point is that centrifugation was not necessary for the separation of phases, but only the addition of another portion of acetone. The repeatability of the extraction, measured as the relative standard deviation (RSD) was less than 11 % [29]. A promising method for extraction of PAHs is via application of gold nanoparticles (20 nm). A mixture of nanoparticle solution and sample is centrifuged at 1400 rpm and then a mixture of pentanethiol and octane is added to the separated solid. Short shaking and centrifugation (13,400 rpm) gives the extract [30].

Headspace Extraction Headspace (HS) extraction is a well-known method of sample preparation and is frequently used in many laboratories, especially in industrial applications. It involves a partitioning equilibrium between the gas phase and a sample (liquid or solid). In this technique, an aliquot of gas phase is sampled into GC. There are two types of analysis, static and dynamic. In the static version, when the equilibrium is reached, the gas phase is injected into GC. In dynamic analysis, the volatiles are exhaustively extracted by the stream of gas. However, matrix effects result in decreased sensitivity for certain substances, especially polar and hydrophilic samples. A comprehensive book describing HS techniques was presented by Kolb [31].

In order to increase the concentration of analytes in the gas phase, the following modifications have been carried out:

- Increasing the incubation temperature
- Addition of salt
- Changing the pH

A static headspace is frequently used for the determination of VOCs in complex matrices such as food [32, 33], urine [34], blood [35], and swimming pool water [36]. This method is also routinely used in the analysis of residual solvents in the pharmaceutical industry [37]. Currently, there are various types of automatic HS systems (Fig. 14.5) [38].

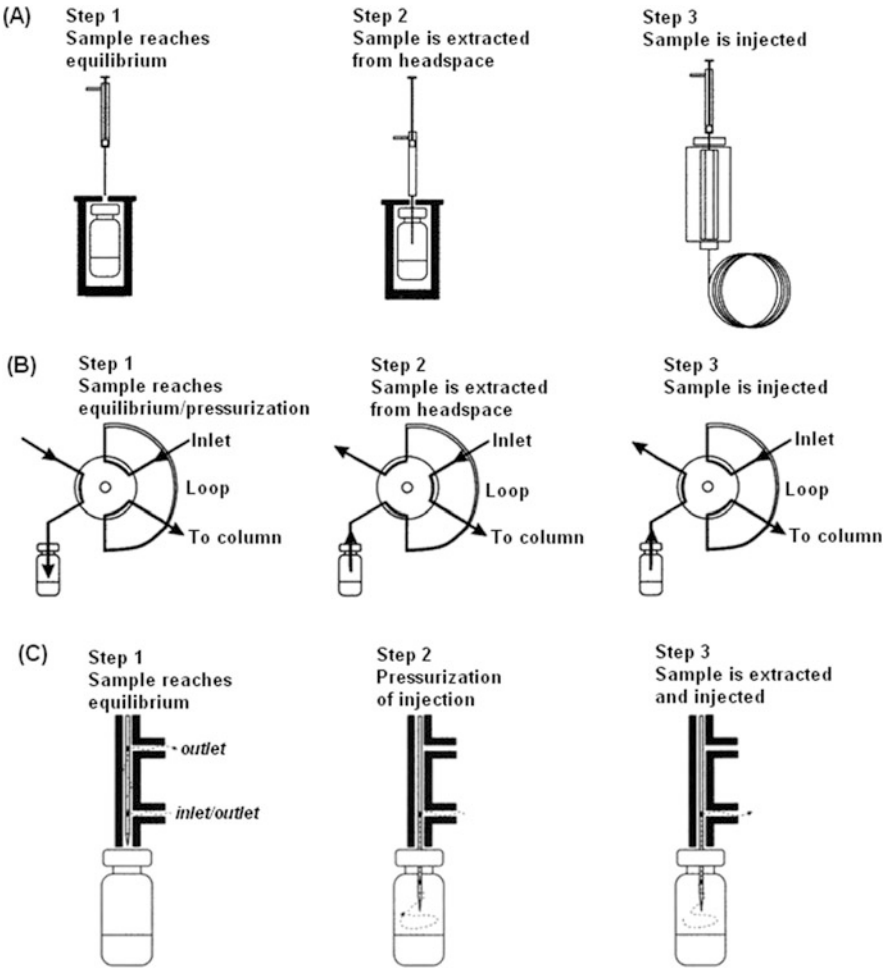

Fig. 14.5 Scheme of headspace systems [38]

Purge and Trap Dynamic headspace analysis is commonly called purge and trap (P&T). The sample is purged by a stream of inert gas, usually helium or nitrogen. VOCs are continually extracted and moved to the sorbent trap. Afterwards, the trap is heated and flushed with carrier gas. The released volatiles are transferred into GC. In addition, the desorption of VOCs is often combined with cryo-focusing prior to injection into the column [39]. The gas stream passing through the sample increases the mass transfer in the system. It provides increased speed and extraction efficiency compared with static HS analysis. The efficiency of the extraction is related to the following factors:

- Polarity of the substance and its solubility in water
- Temperature and ionic strength of the aqueous solution
- Volume of gas used for extraction
- Breakthrough volume of sorbent in the trap

The P&T technique is frequently used for determination of volatiles in food and beverages [40, 41], plants [42], natural water and wastewater [43, 44], sediments [45], and cow slurries [46]. The technique is widely recommended by the EPA.

Closed Loop Stripping Analysis In general, closed loop stripping analysis (CLSA) is similar to P&T, except that the gas is pumped out in a closed circuit via the extraction vessel and a trap containing sorbent. The volatiles are usually trapped on activated charcoal. Following purging, the volatiles are manually eluted from the charcoal trap with an organic solvent (typically carbon disulfide) [47]. An important advantage of CLSA is ultrasensitivity. Such a method has been applied for extraction and determination of VOCs in ground water [48] and tobacco molds [49].

Thermal Desorption Thermal desorption is an alternative GC inlet system particularly used for VOC analysis. However, the analytes subjected to thermal desorption must be thermally stable to achieve successful analysis. Otherwise, decomposition occurs. This technique is mainly used for determination of volatiles in the air. Such a methodology requires sample collection onto solid sorbents, then desorption of analytes and GC analysis. Traditionally, activated charcoal was used as a sorbent followed by extraction with carbon disulfide. However, solvent desorption involves re-dilution of the VOCs, thus partially negating the enrichment effect. Therefore, the sampling method is to pump a sample of gas (air) through the sorbent tube containing certain sorbents in order to concentrate the VOC. Afterwards, the sample tube is placed in thermal desorber oven and the analytes are released from the sorbent by application of high temperature and a flow of carrier gas. Additionally, desorbed compounds are refocused in a cold trap and then released into the GC column. Such a two-step thermal desorption process provides a narrow chromatographic band at the head of the column.

The efficiency of thermal desorption is affected by temperature, desorption time, and gas flow rate. On the other hand, the thermal stability of the sorbents limits the maximum temperature of thermal desorption. The efficiency of cold trapping is further enhanced by filling the trap with a small amount of a certain sorbent.

An important issue, especially in the analysis of the air, is the use of appropriate sorbent materials. The adsorbent must be able to trap target compounds and then release it efficiently. Selection of a suitable absorbent material depends on the following properties [50]:

- Thermal stability
- Surface area
- High breakthrough volume (BTV)
- Low affinity to water
- Molecular weight and volatility of the analytes

Table 14.1 Properties of sorbents [51, 52]

Sorbent	Typical application	Temperature limit (°C)	Specific surface area (m^2/g)	Comments
Tenax TA™ Tenax GR	n-C_7 to n-C_{20}	350	15–25	Low adsorption capacity, application limited to relatively high concentrations, often used in multibed configuration
Chromosorb106	n-C_5 to n-C_{12}	250	750	Less stable than Tenax, not suitable for semivolatiles and multibed
Carbotrap C™	n-C_8 to n-C_{30}	400	12	For multibed, relatively hydrophobic, semivolatiles and alkyl benzenes (BTEX)
Carbopack B™	n-C_5 to n-C_{12}	400	100	Relatively hydrophobic, might be used for trapping of ketones and alcohols
Spherocarb	n-C_1 to n-C_5	400	1200	Last section of multibed, efficient for traces and very volatile organic compounds (VVOCs), problem with humid samples
Carbosieve S III Carboxenes	Mainly gases	400	800	Strong or irreversible sorption of heavier molecules

There are a variety of sorbents that can be used for thermal desorption. Table 14.1 shows some common adsorbents and their properties [51, 52].

Many porous organic polymers are derived from the stationary phase used to pack GC columns. Tenax is one such example. This is a macroporous polymer obtained from diphenyl *p*-phenylene oxide (DPPO). Generally, this polymer is hydrophobic and does not retain water. However, it exhibits some ability to adsorb polar compounds. As a result of its low surface area (30 m^2/g), its adsorption capacity is limited and very volatile compounds are not trapped. Therefore, it is an appropriate material for trapping heavier compounds with more than four carbon atoms. Co-precipitated graphitized carbon black and Tenax (in the proportion 23 % to 77 %) was introduced on the market as Tenax GR. This adsorbent combines the advantages of both materials and is approximately twice as effective as Tenax TA [50].

Tenax is also suitable for use in the cryogenic trap of thermal desorbers. One of the disadvantages of Tenax is its decomposition in oxidative conditions, such as in the presence of ozone and nitrogen oxides. As a result of reaction with ozone, acetophenone and benzaldehyde are generated, and 2,6-diphenyl-p-benzoquinone in the reaction with nitrogen oxides.

Adsorption tubes filled with one type of adsorbent are often unsuitable for simultaneous analysis of a wide range of compounds with varying degrees of volatility and polarity. The solution to this problem is the application of adsorption tubes containing different layers of sorbents (multibed trap) [53]. Such a trap is

usually packed with two layers. The first layer at the front of the tube contains light adsorbents, which trap heavier compounds. The second layer contains stronger adsorbents for more volatile compounds. During the sorption, analytes pass through the tube in the direction of increasing strength of sorption. The least volatile substances are retained on the weakest sorbent located at the inlet of the tube, the more volatile substances are adsorbed on the stronger sorbent, and the most volatile are retained by the strongest adsorbent at the end of the tube. Desorption occurs in the opposite direction to the gas flow to avoid retention of heavy analytes on the strongest sorbent [54].

Typical applications of thermal desorption are related to determination of volatiles and semivolatiles in air samples [55–58]. This technique has been applied for investigation of insect pheromones [59] and drugs in urine [60]. The same principles can be applied for solids or semisolids (soil, sediment, pharmaceutical raw materials, cream, ointments, polymers, etc.)

In the first step, the material located in the tube is heated in the stream of carrier gas and VOCs are released. Simultaneously, the volatile components are focused on a cold trap and later thermally desorbed with carrier into the GC. Thermal desorption is a useful technique, not only for analysis of air pollutants, but also in the case of ambient particulate matter [61].

Needle Trap Device Particularly interesting is development of the needle trap device (NTD) in an attempt at miniaturization of the sorption traps used in the analysis of gas samples. A sorbent bed positioned inside a needle (mounted in a gas-tight syringe) acts as a sorption trap. Different materials such as polydimethylsiloxane (PDMS), carboxenes, carbopacks, Tenax, divinylbenzene (DVB), and other polymers have been employed as sorbents.

During sampling, the gaseous sample is aspirated through the needle containing the sorbent bed, usually by moving the plunger of the syringe. Subsequently, the plunger is moved down, pulling the sample through the needle. After several cycles, the volatile compounds are released from the packed needle in the hot GC injector [62]. The inert gas from the syringe enhances desorption and transfer of analytes from the sorbent. The NTD set-up is presented in Fig. 14.6. Such a system can be operated manually or automatically by robotic autosamplers.

Applications of NTDs are mainly focused on the analysis of gaseous samples and biomedical analyses [64–68]. An important development direction is derivatization in the needle to enhance sensitivity [69]. NTD combines the advantages of SPME with the sensitivity of traditional sorbent traps. However, the main drawback is low reproducibility of the needle packing.

Solid Phase Microextraction Out of the many sample preparation methods, solid phase microextraction (SPME) is one of the most frequently used. SPME is used for the determination of VOCs in liquid, gas, and solid samples. The great advantage of the method is that it combines, in one stage, the isolation and enrichment of compounds, and completely eliminates the need for organic solvents.

Hence, SPME offers a simple and inexpensive alternative to the various extraction methods. SPME was developed in the late 1980s by Arthur and Pawliszyn [70].

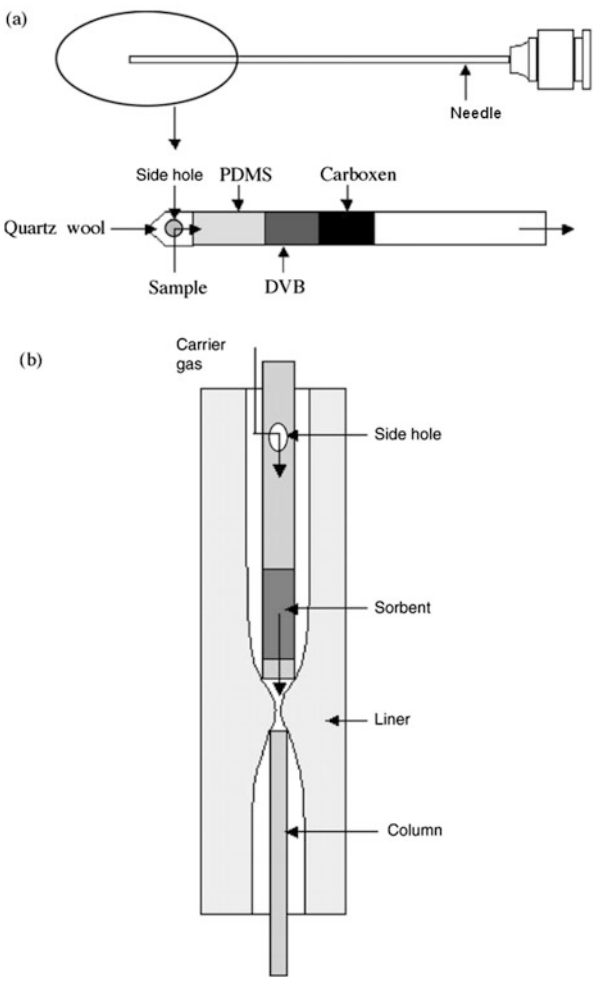

Fig. 14.6 Scheme of a needle trap device: (**a**) needle, (**b**) desorption in liner [63]

This method utilizes a fused silica rod coated with a thin layer of stationary phase mounted in a holder. During extraction, the fiber is exposed to the sample, and analytes are adsorbed onto the stationary phase and concentrated. After a defined extraction time, the fiber is withdrawn in the holder and then analytes are thermally desorbed in the GC injector. Several types of coatings are commercially available, such as PDMS, polyamide, Carbowax-DVB, Carboxen-PDMS, and PDMS-DVB. Coatings prepared with three kinds of materials are also available (e.g., DVB-Carboxen-PDMS) [71]. Selection of the fiber is mainly based on the principle "like dissolves like." For example, PDMS sorbent is suitable for the extraction of hydrocarbons, and the sorbent should be polar for the extraction of alcohols or ketones. The thickness of the coating film determines the sorption capacity of the fiber. Changing the temperature, pH, or ionic strength of the liquid

sample can affect the value of the distribution constant and thereby increase the efficiency of extraction.

As a result of the physicochemical properties of certain groups of analytes (e.g., carbonyl compounds), and their low concentration levels, it is necessary to transform the analytes to derivatives that are less polar and have higher volatility and thermal stability. This process improves extraction efficiency and detection. There are several approaches to derivatization in combination with SPME; however, on-fiber derivatization seems to be the most suitable. Prior to extraction, the fiber is loaded with derivatization agent. Afterwards, the fiber is exposed to the sample and analytes are simultaneously extracted and converted to derivatives. An example of such methodology is the analysis of formaldehyde, which involves formation of an oxime with high affinity to the sorbent layer [72]. The typical reagents used for derivatizing the carbonyl groups (aldehydes, ketones) are 2,4-dinitrophenylhydrazine (DNPH), *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA), and 2,3,4,5,6-pentafluorohydrazyna (PFPH).

In order to increase the selectivity and efficiency of extraction, novel stationary phases have been developed, particularly in trace analysis. Cyclodextrins, graphitized carbon blacks, and conductive polymers such as polypyrrole and polyaniline have been investigated [73]. Sol–gel polymers also seem to be interesting. A particularly important feature is the high thermal stability of these polymers [74]. Recent applications for analysis of biological samples have been described in several articles [75–81]. Review articles present recent developments in methodology, the SPME technique [82–84], and novel coatings [85, 86].

Dynamic Solid Phase Extraction The technique of dynamic solid phase extraction (SPDE) can be regarded as similar to NTD, where the inner wall of the needle is coated with polymer. Lipinski introduced such a concept and attached a short section of metal capillary column coated with typical PDMS polymer to a gas-tight syringe [87]. The SPDE system is presented in Fig. 14.7 [88].

The sample (liquid or gas over the sample) is aspirated into the syringe. Afterwards, the syringe plunger is moved down, pulling the sample through the needle. Such a cycle is repeated several times. The analytes passing through the needle are absorbed in the PDMS layer. After extraction, the syringe is moved to the heated GC injector and analytes are thermally desorbed.

Followed placement of the needle, desorption using a stream of helium is realized in a hot injector GC. Gas from the syringe enhances desorption and results

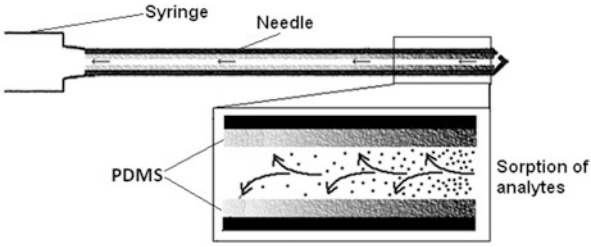

Fig. 14.7 Dynamic solid phase extraction concept; *PDMS* polydimethylsiloxane [88]

in a narrow chromatographic band. A model describing the kinetics of extraction of VOCs by means of this technique has been presented [88]. The main advantages of SPDE are greater durability of polymer and better sensitivity in comparison with SPME. Recently this technique has been automated and commercialized. This technique was recently reviewed [89–91].

Stir-Bar Sorptive Extraction This technique was developed to overcome the problems related to the very small amount of fiber coating material that is applied in SPME. Stir-bar sorptive extraction (SBSE) utilizes a magnetic rod coated with polymeric sorbent. Such a concept was introduced by Baltussen [92]. The magnetic stir bars have a length of 1 or 2 cm, and the polymer film a thickness of 500 or 1000 μm. The bar provides a large volume of polymer coating and its sorption capacity is much higher than in the conventional SPME method. In this technique, the sorbent-coated stir-bar is exposed directly to the liquid sample. When equilibrium is reached, the stir-bar is placed in the thermal desorber unit and analytes are released into GC.

SBSE can be successfully used in the analysis of environmental samples [93–97] and for food analysis [98, 99]. PDMS is the most commonly used polymer, primarily because of its thermal stability and durability. SBSE has been modified by application of derivatization with different reagents (acetic anhydride, BSTFA, etc) [100–104]. This approach is suitable for the extraction of compounds requiring derivatization. The use of multistep derivatization with several extraction elements (each reaction is performed on a different stir bar) allows efficient extraction, desorption, and chromatographic analysis of compounds with different functional groups (e.g., phenols, steroids, amines, thiazoles, ketones). Acetic anhydride (ester formation), ethyl chloroformate (reaction of acids and amines), tetraethyloborane, and sodium bis-trimethylotrifluoroacetamide have been used for extraction and simultaneous derivatization [105].

Membrane Techniques The interest in membrane techniques for sample preparation arose in the 1980s. Extraction selectivity makes membrane techniques an alternative to the typical sample enrichment methods of the 1990s. Different membrane systems were designed and introduced into analytical practice; some more prominent examples are polymeric membrane extraction (PME), microporous membrane liquid–liquid extraction (MMLLE), and supported liquid membrane extraction (SLME) [106, 107]. Membrane-assisted solvent extraction (MASE) coupled with GC-MS is another example of a system that allows analysis of organic pollutants in environmental samples [108–111]:

Membrane extraction with sorbent interface (MESI) is an interesting example of an extraction device, which is the most useful system for interfacing with GC. In this approach, the donor phase is a gas or a liquid sample, and the acceptor phase is a gas. The volatiles are continuously trapped on sorbent and then desorbed into GC [112]. Another solution is a combination of off-line GC-MESI through a cryogenic trap, which allows preparation of environmental samples in the field and performance of GC analysis after transportation to the laboratory [113, 114]. MESI allows the extraction of volatile and relatively nonpolar analytes.

A consequence of MESI application is the membrane introduction mass spectrometry system (MIMS), which allows selective "extraction" of VOCs and direct MS analysis without chromatographic separation. The gaseous substances permeate through the highly hydrophobic membrane and are introduced directly to a mass spectrometer [115–118].

14.3 Methods for the Detection of Volatile Organic Compounds

Separation of VOCs is usually performed by GC, because it allows efficient separation of compounds. Nowadays, GC capillary columns are high performance and thermally resistant. Separation of VOCs is done by columns of high thermal stability containing effectively crosslinked polymer that is chemically bonded to the surface of the capillary.

14.3.1 Gas Chromatography and Mass Spectrometry

Traditional detectors (i.e., FID; electron capture detector, ECD; nitrogen-phosphorous detector, NPD) supply only retention data. However, in many cases this is not enough for proper identification of analytes. Application of GC coupled with an MS detector gives much more information (i.e., the mass spectrum of each compound). GC-MS is a well known and frequently used technique that combines the highly effective separation of GC with the high sensitivity and selectivity of MS. Moreover, improvements in analytical instruments based on different types of mass analyzers (ion trap, quadrupole, and TOF) and the development of hybrid Q-TOF has enhanced the analytical capabilities of modern hardware. Different kinds of mass spectrometers are presented in Table 14.2 [119].

Typical MS detectors enable identification of compounds in the case of analyte co-elution. Another convenience is the widespread use of commercial libraries of spectra (e.g., NIST, Wiley), which are useful in the qualitative analysis of complex mixtures. It is widely believed that GC-MS is the most appropriate tool for identification of VOCs in different matrices.

However, the desire to reduce or even eliminate the required sample preparation has led to the development of newer techniques, such as proton-transfer-reaction mass spectrometry (PTR-MS) and selected ion flow tube mass spectrometry coupled with ion mobility spectrometry (MS-SIFT IMS). These techniques allow real-time measurements within a single second. This enables analysis of gas composition during technological processes, monitoring of indoor air, and investigation of VOCs emitted by living organisms.

Table 14.2 Selected parameters of common mass analyzers [119]

Analyzer/parameter	Quadrupole (Q)	Ion trap (IT)	Time-of-flight (TOF)
Mass range (Da)	2–4000	10–2000	No limit
Resolution	Nominal	Nominal	High
Mass accuracy	1 Da	1 Da	<1 ppm
Limit of detection (g)	10^{-13}–10^{-12}	10^{-13}–10^{-12}	–
Linearity	4–5	3–4	3–5
Scanning speed (Hz)	50	50	1000

14.3.2 Proton-Transfer-Reaction Mass Spectrometry

PTR-MS is a relatively new technique for direct analysis of VOCs. It was first used for the direct analysis of a mixture of gases in 1995 by the research group of Hansel [120]. The principle of this system is introduction of steam to the ionization area and formation of reactant ions (H_3O^+). These ions are generated using a hollow discharge cathode or under the action of α-radiation emitted by isotope ^{241}Am [121]. High purity reactant ions helps to increase the sensitivity of the method and eliminates the need to apply a quadrupole to select reaction ions prior to their entry to the reaction area. Together with H_3O^+, O_2^+ ions might also be present because they are formed as a result of charge transfer between H_3O^+ and O_2.

The reaction ions enter the area where the carrier gas introduces the sample. H_3O^+ ions rapidly react with the analyte molecules (R), on the basis of the proton transfer reaction. As a result of this process, analyte cations (RH^+) are produced. RH^+ ions enter the drift region, where they are separated in the electric field according to their mass-to-charge ratio (m/z):

$$H_3O^+ + R \rightarrow RH^+ + H_2O$$

As reactive ions, NH_4^+ cations can be used. However, most VOCs have a great ability to join with a proton from H_3O^+. That is why almost every collision is effective and leads to formation of the reaction product, a cation. Exceptions are alkanes of low molecular weight that reveal low proton affinity. In these conditions, dissociation with proton transfer takes place, leading to a reaction product mixture [122]. For example, ionization of sevoflurane ($C_4H_3F_7O$) gives various products, and the processes proceed with different yields:

$$\begin{array}{c} C_4F_7OH_2^+ + H_2O + H_2 \quad (79\,\%) \\ H_3O^+ + C_4H_3F_7O \rightarrow C_4F_6H_2OH^+ + H_2O + HF \quad (12\,\%) \\ CHFOH^+ + H_2O + C_3F_6H_2 \quad (9\,\%) \end{array}$$

Leaving the drift area, ions enter the analyzer where they are separated. Figure 14.8 shows a scheme of the PTR-MS system [123]. Some difficulties appear when a complex mixture is analyzed, because the ionization of isomers by proton

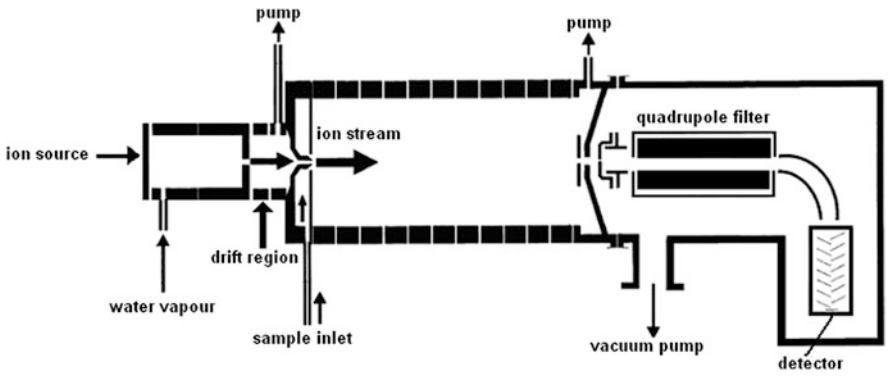

Fig. 14.8 Scheme of proton-transfer-reaction mass spectrometry system [123]

transfer pseudomolecular ions gives identical values of *m*/*z*. This means that application of PTR-MS for identification of chemical substances is limited. It cannot be used for identification of unknown substances. Therefore, PTR-MS is a good technique for direct monitoring of substances whose presence has been confirmed by other methods [124].

Application of PTR-MS for analysis of gaseous mixture avoids enrichment, preconcentration, and preseparation processes because of the high sensitivity of the system (ppb to ppt). PTR-MS is used for analysis of VOCs in exhaled air, because although substances such as CO_2, N_2, O_2, and H_2O are present in the sample at very high concentrations, they do not interfere with the measurement. The advantage of this technique is the short time of analysis, which typically takes only a few seconds. A number of publications have appeared in the last 15 years describing the use of PTR-MS as a noninvasive diagnostic method based on the direct analysis of exhaled air. This method was used to study changes in the emission of VOCs released from coffee, meat, and fruit by cultures of bacteria [125].

14.3.3 Selected Ion Flow Tube Mass Spectrometry

Selected ion flow tube mass spectrometry (SIFT-MS) is an analytical technique used for direct and quantitative determination of VOCs in mixtures of gases. SIFT-MS was introduced in 1976 by N. G. Adams and D. Smith. The technique can be applied for parallel real-time monitoring of a few substances [126]. A scheme of the SIFT-MS system is presented in Fig. 14.9 [127]. The principle of gas mixture analysis is based on the reaction of reagent ions with molecules of analyte within a specific time (a few milliseconds). In this method, chemical ionization is applied; reagent ions are generated in the ion source by a suitable ionization gas (nitrogen, oxygen, or water vapor). Of all the obtained ions, only cations of the desired *m*/*z*

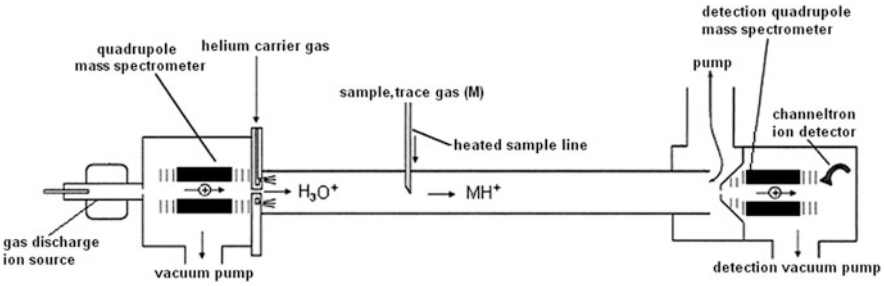

Fig. 14.9 Scheme of selected ion flow tube mass spectrometry system [127]

ratio are selected by the quadrupole filter. Subsequently, the selected ions are introduced into the reaction stream of inert carrier gas, usually helium, and enter the drift tube. The flow channel is a tube of 30–100 cm in length, the pressure is approximately 100 Pa, and the temperature is 300 K. The sample is introduced into the tube via a thin capillary. The speed of sample introduction has to be properly adjusted. In order to prevent condensation of analytes in the capillary it is heated to 100 °C.

Reagent cations such as H_3O^+, NO^+, or $O_2^{+\bullet}$ react very fast with molecules of analyte. As a result of this process, specific cations of analyzed compounds are produced (MH^+):

$$H_3O^+ + M \rightarrow MH^+ + H_2O$$

The number of reaction ions decreases as the number of analyte molecules increases. The population of pseudomolecular ions is described by the kinetic equation:

$$[MH^+]_t = [H_3O^+]k[M]\,t,$$

where $[MH^+]_t$ is the concentration of protonated cation of analyte at time t, t is the time of reaction of precursor ion with analyte molecule, [M] is the concentration of analyte in gas phase, and k is the rate constant for reaction of precursor ion with analyte molecule.

The rate constants for reaction of precursor ions H_3O^+, NO^+, and $O_2^{+\bullet}$ with different molecules were determined experimentally. To calculate the partial pressure of the analyte [M] it is necessary to know the value of constant k for each reaction and the concentration of $[MH^+]$. The method also allows simultaneous measurement of the concentration of several analytes. Studies have shown that ions such as H_3O^+ and N^+ react quickly enough with various organic compounds, but $O_2^{+\bullet}$ reacts quickly only with small molecules such as NO, NO_2, or NH_3. In the case of samples containing a large amount of moisture (e.g., breath samples or headspace of aqueous solution), cluster ions such as $H_3O^+(H_2O)_{1,2,3}$ may be formed in

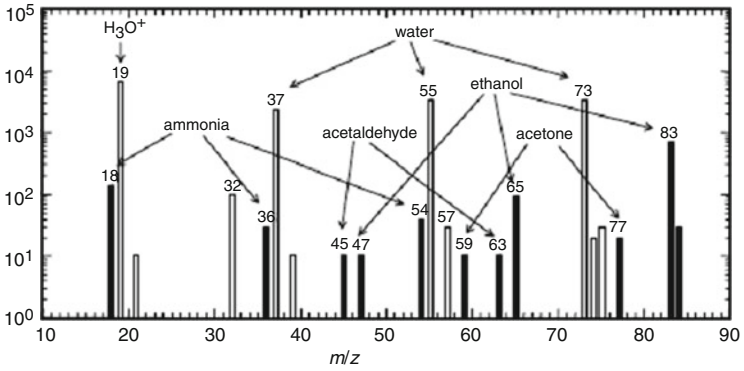

Fig. 14.10 Mass spectrum of volatile organic compounds in expired air, obtained using selected ion flow tube mass spectrometry. H_3O^+ ions were applied as reagent ions [127]

addition to H_3O^+ ions. These clusters react with the analyte molecules to give cations of the water molecules, $MH^+(H_2O)_{1,2}$:

$$H_3O^+(H_2O)_{1,2,3} + M \rightarrow MH^+(H_2O)_{1,2} + H_2O$$

Various kinds of analyte cations and reaction ions formed during the reaction hit the MS analyzer and are separated according their *m*/*z* ratio. As a result of the measurements, mass spectra of the analyzed compounds are obtained.

Using SIFT-MS it is possible to record mass spectra in the full-scan mode (FS) of quadrupole MS or in multiple ion monitoring mode (MIM) when ions are passed thorough the quadrupole with a certain *m*/*z* ratio. FS mode allows the full mass spectrum of analytes in a defined range of *m*/*z* values to be registered within a specified time period. In the case of MIM, precursor ions and selected ions, formed during reaction of the reagent ion and the analyte, are scanned.

SIFT-MS was used for the analysis of VOCs present in air samples [127]. Substances such as ammonia, isoprene, acetone, ethanol, and acetonitrile were detected at ppb levels. SIFT-MS was also applied to analysis of blood [126], urine [128], food [129], and headspace from lung cancer cells [130]. The spectrum of exhaled air is presented in Fig. 14.10.

14.3.4 Ion Mobility Spectrometry

Ion mobility spectrometry (IMS) is often coupled with GC and applied for VOC analysis. IMS identifies chemical substances based on their ion speed in the gas phase. These ions drift under the influence of applied voltage (100–350 V/cm) [131]. The detector combines the low cost of a single analysis with the short time necessary to obtain a measurement result (20–50 ms) and a low detection limit for

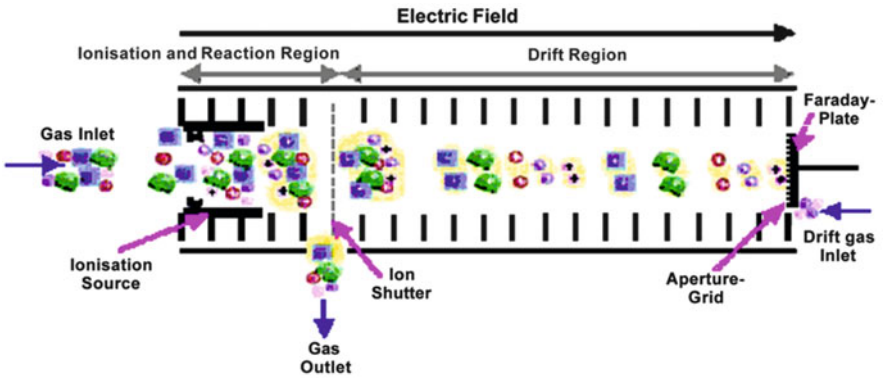

Fig. 14.11 Scheme of separation in ion mobility spectrometry detector [132]

certain classes of chemical compounds. Aldehydes, ketones, amines, and esters are detected at the nanogram per cubic decimeter level. Note that using IMS it is difficult to detect VOCs with low proton affinity (i.e., alkanes and aromatic hydrocarbons). The IMS detector is easy and convenient to operate. However, its disadvantage is low selectivity for analytes in a complex matrix. Therefore, in practice it is often combined with column chromatography (usually multicapillary, MCC) for preliminary separation of analytes. Substances eluted from the multicapillary column reach the ion source, where the carrier gas (e.g., nitrogen, synthetic air) undergoes ionization by β-radiation (^{63}Ni, ^{3}H). Then, analyte ions are produced by collisions of carrier gas ions with analyte molecules. A scheme of ion separation in the drift tube of IMS is presented in Fig. 14.11 [132].

Another ionization method is application of corona discharge and photoionization by laser or UV light. The ionization area is separated from the drift area by a partition, which is periodically opened. When the partition is open, ions penetrate the drift region and undergo separation according to their m/z ratio. If the ion compartment is closed, ions are removed from the system. The first ions to reach the detector (the Faraday plate) are the lightest ions with the greatest charge, followed by heavier ions. Ions are collected on the Faraday plate, and the signal is amplified and transmitted to the recorder. Nitrogen gas is supplied to the end part of the drift region and transfers the unionized particles to the area of ionization. The result of analysis is a drift time spectrum, which is the relation between current detected by the detector and the drift time of separated ions. Drift time is characteristic of a specific ion under specific conditions. If the measurement system consists of GC for analyte preseparation and IMS as detector, there are two parameters that characterize measured substances: retention time and drift time.

In the IMS detector, it is possible to apply a positive or negative polarity. In the first step, the carrier gas during ionization gives reactant ions, which then collide with analyte molecules causing their ionization. Initially, a fragile cluster ion is formed that, after elimination of a water molecule, forms a more stable ion, the

reaction product. An increased number of cluster ions is also formed when the analyzed sample contains a significant amount of water vapor.

Positive polarization:

$$\underset{\substack{\text{analyte}\\\text{product}\\\text{molecule}}}{\mathrm{M}} + \underset{\text{reagent ion}}{\mathrm{H^+(H_2O)}_n} \rightarrow \mathrm{MH^+(H_2O)}_n \rightarrow \mathrm{MH^+(H_2O)}_{n-x} + \underset{\text{cluster ion}}{x\mathrm{H_2O}}$$

Negative polarization:

$$\underset{\substack{\text{analyte}\\\text{molecule}}}{\mathrm{M}} + \underset{\text{reagent ion}}{\mathrm{O^{2-}(H_2O)}_n} \rightarrow \mathrm{MO^{2-}(H_2O)}_{n-x} + \underset{\text{product}}{x\mathrm{H_2O}}$$

If the analyte concentration is too high, there is a high probability of collision between the ion product formed (monomer) and a nonionized analyte molecule, resulting in dimer formation. At very high concentrations of the measured substances, it is possible that there are too few reaction ions and therefore some of the analyte molecules are not ionized:

$$\underset{\substack{\text{protonated}\\\text{monomer}}}{\mathrm{MH^+(H_2O)}_n} + \mathrm{M} \rightarrow \underset{\substack{\text{analyte}\\\text{molecule}}}{\mathrm{M_2H^+(H_2O)}_{n-x}} + \underset{\text{dimer}}{x\mathrm{H_2O}}$$

GC coupled with IMS has many advantages and is applied for quality control in the pharmaceutical industry [131, 132] and for detection of trace amount of explosives, warfare agents, and drugs [133–135]. This system is used to monitor ambient air [136] and for direct analysis of exhaled air [137].

14.4 Conclusions

Gas chromatography is the technique of choice for analysis of VOCs. Its combination with MS in various configurations is now indispensable in the identification of compounds. The development of TOF MS and comprehensive two-dimensional gas chromatography (GC×GC) have revolutionized accurate measurement with high resolution spectrometers. However, sample preparation is a crucial stage prior to analysis. Therefore, development of real-time measurement methods such as PTR-MS, SIFT-MS, and IMS for direct measurement of VOCs (especially in gases) is very valuable for instrumental analysis. It should also be noted that there is still interest in developing sample enrichment techniques, especially for polar compounds. The application of nanoparticles, ferromagnetic sorbents, molecular imprinted polymers, and ferromagnetic ionic liquids should be also noted.

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Chapter 15
Analysis of Radionuclides

Bogdan Skwarzec

15.1 Introduction

15.1.1 Radioactivity and Accompanying Phenomena

Radioactivity is the ability of a substance to emit alpha, beta, and gamma radiation as a result of atomic nucleus decay. The nuclei of radioisotopes are unstable and the following transformations might occur in them:

(a) Alpha decay (α): emission, by the nucleus, of alpha particles consisting of two protons and two neutrons (a helium nucleus).
(b) Beta-minus decay (β^-): emission of electrons during the transformation of neutrons into protons inside an atomic nucleus with an excess of neutrons.
(c) Beta-plus decay (β^+): emission of positrons during the transformation of protons into neutrons in an atomic nucleus with an excess of protons.
(d) Electron capture (EC): absorption of electrons with a K- or L-shell by an atomic nucleus; the absorbed electron combines with a proton to yield a neutron, neutrino, X-ray, γ-ray, Auger electrons, and inner "bremsstrahlung."

In addition to the above transformations, the following accompanying phenomena can occur in an atomic nucleus:

(a) Emission of gamma radiation (γ)
(b) Internal conversion
(c) Emission of X-rays (Röentgen) [1].

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15.1.2 Natural and Artificial Radioactivity

There are three names connected with the discovery of radioactivity: Henry Becquerel, who discovered this phenomenon in 1896 [2]; Maria Skłodowska-Curie, who named this process "radioactivity"; and her husband Pierre Curie [3]. They stated that uranium salts emit ionizing rays and, furthermore, Maria Skłodowska-Curie discovered that thorium gives off the same rays. She proved that radiation was not the outcome of some interaction of molecules, but must come from an atom itself; this discovery was absolutely revolutionary. Maria and Pierre discovered the first two radioactive elements, polonium and radium. There are about 20 radioactive elements and about 50 radionuclides in the natural environment.

The first artificial radionuclide, ^{30}P, was produced in 1934 by Frédéric and Iréne Joliot-Curie (daughter and son-in-law of Maria Skłodowska-Curie) by bombarding aluminium with protons in an accelerator [4]. Today, more than 2000 artificial radionuclides have been produced and identified, especially after the discovery and use of nuclear fission of uranium ^{235}U and plutonium ^{239}Pu.

The number of radioactive nuclei with a time function is smaller. The half-life is characteristic for each radionuclide and is defined as the period during which half of the number of radioactive nuclei decay. Every radionuclide decays according to equation (15.1):

$$A_0 = A_t \cdot \exp\left[\frac{t \cdot \ln 2}{T}\right] \tag{15.1}$$

where A_0 is the activity of the radionuclide at time $t = 0$, A_t the activity after time t, T the half-life of the radionuclide, and t the time between 0 and t.

The activity of a radionuclide is the number of decays in a unit of time. Nowadays, the becquerel (1 Bq = 1 decay per second) is the unit of radioactivity, but in the past it was the curie ($1\ \text{Ci} = 3.7 \times 10^{10}$ Bq).

Radionuclides present in the environment are classified as being either of natural or artificial (anthropogenic) origin [1]:

1. Naturally occurring radionuclides
   (a) Radionuclides of terrestrial origin (e.g., ^{40}K and ^{87}Rb)
   (b) Cosmogenic radionuclides (e.g., ^{3}H, ^{14}C, ^{32}Si, and ^{36}Cl)
   (c) Primary radionuclides: These long-lived radionuclides have been ubiquitous on the Earth ever since its formation. The radionuclides ^{238}U, ^{232}Th, and ^{235}U are the respective parent members of the uranium, thorium, and actinium radioactive decay series
2. Artificial (anthropogenic) radionuclides
   (a) Neutron activation products (e.g., ^{22}Na, ^{54}Mn, ^{55}Fe, ^{60}Co, ^{63}Ni, ^{64}Cu, ^{65}Zn, ^{110m}Ag, ^{124}Sb, and ^{125}Sb)

(b) ^{235}U and ^{239}Pu fission radionuclides (e.g. ^{90}Sr, ^{95}Zr, ^{131}I, ^{132}I, ^{132}Te, ^{137}Cs, and ^{144}Ce)
(c) Transuranic elements (e.g. ^{238}Pu, ^{239}Pu, ^{240}Pu, ^{241}Pu, ^{241}Am, and ^{243}Am)

Taking into account their half-life, type of decay, and strong radiotoxicity, the most important radionuclides in the natural environment are ^{210}Po, ^{210}Pb, ^{222}Rn, ^{226}Ra, ^{234}U, ^{235}U, and ^{238}U. Important artificial radionuclides are ^{55}Fe, ^{60}Co, ^{63}Ni, ^{90}Sr, ^{137}Cs, ^{238}Pu, ^{239}Pu, ^{240}Pu, and ^{241}Pu. In the natural environment these radionuclides occur in trace quantities [1].

15.1.3 Radiometric Methods

Detectors are a very important element of radiometric measuring instrumentation and are used to measure activity based on the ionizing process in gases, liquids, and solids. The following detectors are used to measure ionizing radiation [5]:

(a) Gaseous detectors: ionizing cells, proportional counters (for measuring α and β^- particles), and Geiger–Müller counters (especially for β^- particles)
(b) Scintillation detectors: crystal, plastic, and liquid scintillators (in a scintillator the exchange of ionizing radiation for light quanta occurs)
(c) Semiconductor detectors not only count the ionizing particles, but can also act as spectrometers to measure spectra. Detectors with silicon and germanium monocrystals are used to measure alpha and gamma radiations.

Low-level alpha, beta, and gamma spectrometry is used to determine low levels of activity of radioactive substances in the environment. Considering the nonlinear character of alpha and gamma spectra, the identification of radionuclides is based on the measurement of energy of γ-quanta or α-particles emitted by isotopes. This allows determination and identification, simultaneously, of some radionuclides in gamma and alpha spectrometry. The reduction of geochemical background in these methods can be obtained by using the coincidence and anticoincidence systems during measurement. In contrast to alpha and gamma spectrometry, the spectrum of emission for β^- electrons is linear and characterized by the average energy (E_{av}) and maximum energy (E_{max}). Thus, the simultaneous determination and identification of some beta-emitting radionuclides is not possible in beta spectrometry, particularly for isotopes of the same element [1].

15.1.4 Gamma Spectrometry

Gamma radiation is the stream of photons with energy between several dozen kilo-electron volts and peta-electron volts; the typical range of gamma radiation in the environment is from 40 keV to about 3 MeV [5].

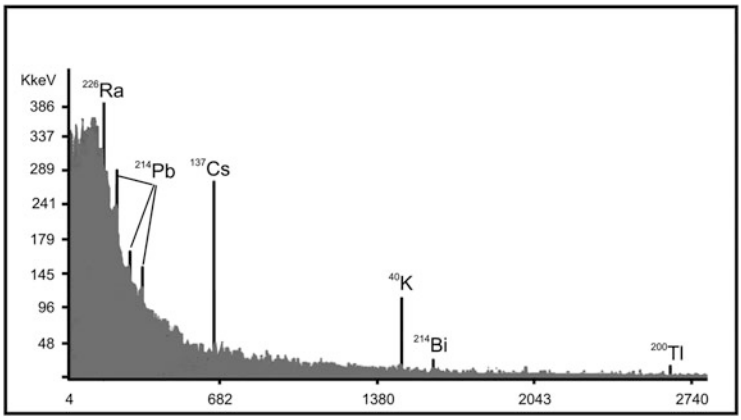

Fig. 15.1 Gamma radiation spectrum [5]

The precise study of gamma spectra requires protection of the measurement area against contaminants, especially fresh air, radon and its decaying products, and materials containing high density elements or metals of high ordinal numbers (e.g., lead, wolfram, copper, and iron) that contain low quantities of radioactive substances (especially U, Ra, Th, K, and the products of their decay). Reduction of the background of the empty gamma spectrometer by enlarging the thickness of the barrier does not guarantee a low detection limit. It is necessary to minimize the intensity registration (voltage) of the diffused radiation of a sample and shift the induction of roentgen fluorescence in the barrier in the energy area not registered by the spectrometer [5].

The utilization of gamma spectrometry for the quantitative determination of low level activity radionuclides in the natural environment is limited because the influence of the natural geochemical background is very strong, but the technique is often used in the study of airborne radioactivity [6, 7]. Low-level gamma spectrometry is used principally to determine the activity of nuclides such as ^{40}K, ^{60}Co, ^{137}Cs, and ^{210}Pb (Fig. 15.1).

15.1.5 Beta Spectrometry

The most accurate method of measuring beta radiation spectra is the electromagnetic method, in which crossed magnetic and electric fields (together with mechanical barriers) are used to choose the energy of electrons or positrons. Electromagnetic spectrometers are not good for analysis of environmental radiation. In such cases, nonspectrometric techniques, such as gas counters and a wide range of liquid scintillation counters, are used for measuring low-energy beta emitters. The preparation containing a radionuclide is mixed with an organic scintillation solution and analyzed in a scintillation counter. Today, this method

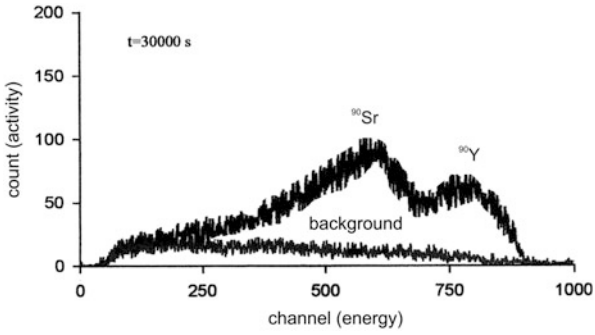

Fig. 15.2 Beta radiation spectra of radioisotopes ^{90}Sr–^{90}Y [5]

is the best known, commonly used, universal and perfect procedure for determining beta radionuclides in environmental samples. Previously, the scintillation cocktail was toluene or benzene containing PPO (2,5-diphenyloxazole) and a substance that shifts the wavelength of emitted light by POPOP [1-4, bis-2(5-phenyloxazolyl) benzene)]. Today, noncombustible scintillators of high chemical stability (e.g. Ultra Gold Canberra or HiSafe 3 Wallac) [5] are in use. A reduction in geochemical background during measurement in this method can be obtained by the use of a 5–10 cm thick lead cover and an additional anticoincidence system with plastic (e.g. Wallac Guardian), liquid (e.g. Wallac Quantulus), or bismuth–germanate crystal scintillators (e.g. Packard-Canberra model 2770). The time of the scintillation process enables differentiation of signals originating from alpha and beta radiations [8]. Recently, it has been possible to use semiconductor detectors to measure beta radiation [9]. The spectrum of beta radiation used by liquid-scintillation spectrometry (Wallac 1414-003 Guardian) is shown in Fig. 15.2.

The activities of some isotopes, in particular ^{90}Sr–^{90}Y, can also be detected by liquid-crystal spectrometry with the use of the Cherenkov phenomenon [10, 11]. The Cherenkov effect is used to determine beta isotopes emitting particles whose E_{max} is above 500 keV [12]. The main advantage of beta activity determination by the Cherenkov effect is the use of analytical preparation used for another chemical analysis (e.g. calculation of recovery). Moreover, the addition of low energy beta or alpha radiation does not disturb the measurement, thereby lowering the cost of analysis. The weakness of this method is the decreased recovery registration and the decline in information about the realistic appearance of the beta spectrum [13]. The determination of beta isotopes in environmental samples is very difficult and requires their chemical isolation. The type of sample and the time of chemical analysis determine the choice of analytical method. Also, the time between contamination and sample collection is important; procedures used for samples recently contaminated are different to those used for old samples in which the decay of short-lived radionuclides has already taken place [1, 5].

15.1.6 Alpha Spectrometry

Alpha spectrometry is one of the most important radiometric techniques used for measuring alpha particles emitted by natural and artificial radionuclides. The alpha spectrometer contains low-level semiconductor detectors with an active surface of 100–600 mm^2, placed in a vacuum chamber connected to a 1024 multichannel analyzer. The detectors used are silicon diodes with a thin gold layer. The efficiency of these detectors is between 25 % and 40 %, but the resolution lies in the range of 20–35 keV. The alpha spectrometer measures alpha particles of energy 3–8 MeV and enables the determination of most alpha radionuclides in the natural environment [1]. Considering the small penetration of alpha particles, the use of alpha spectrometry should precede radiochemical separation of analyzed radionuclides. The environmental samples analyzed are usually mineralized by dry or wet methods using hot concentrated acids (HF, HNO_3, HCl, $HClO_4$) [1, 14].

15.1.7 Neutron Activation Analysis

Neutron activation analysis (NAA) is a sensitive multielement analytical technique used for both qualitative and quantitative analyses of major, minor, trace, and rare elements. The NAA method is based on the transformation of stable nuclides into radioactive nuclides by bombarding the sample with neutrons, followed by measurement of radiation, particularly gamma radiation. Today, artificial radionuclides in samples can be measured in two ways [15, 16]:

1. Destructive method (radiochemical neutron activation analysis, RNAA): based on the chemical separation of radioelements into fractions, each of which contains some radionuclides [17–20].
2. Nondestructive method (INAA instrumental neutron activation analysis): based on the measurement of radioactivity of samples analyzed by high-resolution gamma spectrometry using the differentials of the rate of decay of the radionuclides analyzed in time [15]. NAA has several important advantages [15]:

   (a) Low detection limits for many elements
   (b) Absence or minimization of a black sample
   (c) Multielement analysis
   (d) Nondestructive analysis of dry, solid, or powdered samples
   (e) Good accuracy of determined elements.

NAA is preferred for the analysis of unique samples such as meteorite, the certification of reference materials, and quality assurance in analytical chemistry [21]. "Definitive methods" are based on the activation of samples, selective separation of analyzed radionuclides by column (ion-exchange) chromatography, and measurement by gamma spectrometry [15, 16, 19]. In definitive methods, radiochemical separation, co-precipitation, extraction, and ionic exchange guarantee the

high purity of analyzed radionuclides and high recovery. The separation of radionuclides is usually carried out by adding inactive carriers, and recovery is determined by gravimetric, titration, or colorimetric methods. In definitive methods, separation must be highly selectivity in both quantity and quality, because these methods are intended for the determination of only one element [16].

The application of NAA in trace analysis involves a nuclear reactor, which is the source of a stream of neutrons. The most important advantage of NAA is its nondestructive nature, making it is very useful for analyzing soil, sediment, rock, biological samples, and food. NAA is also used in criminal and judicial toxicology to study the source of intoxication by inorganic (As, Hg) or organic compounds such as halogenoorganic (chloro-, bromo-, and, iodo-organic) compounds present in pesticides and herbicides as well as in marine organisms [22, 23].

15.2 Application of Radioanalytical Methods in Environmental Studies: Analytical Aspects

15.2.1 Activity Measurement of ^{40}K

The isotope ^{40}K is a naturally occurring radionuclide with a long half-life ($T = 1.26 \times 10^9$ years) and is present in natural potassium at 0.0118 %. Potassium ^{40}K is transformed by β^- emission to ^{40}Ca (89.05 %) and by electron capture to ^{40}Ar (10.95 %) [24]. The measurement of ^{40}K activity can be analyzed in environmental samples, after drying and homogenizing, by gamma spectrometry with the use of energy spectrum of 1461 keV. In natural water samples, ^{40}K is measured using the Cherenkov counting technique [25, 26]. Thermal ionization mass spectrometry (TIMS) has also been used to determine ^{40}K in environmental samples [27].

15.2.2 Activity Measurements of Activation Products ^{55}Fe, ^{60}Co, and ^{63}Ni

A number of artificial radionuclides are produced as a result of activation during nuclear weapons tests, operation of reprocessing plants and reactors in nuclear power stations, and in nuclear studies. Modern radioanalytical techniques have enabled activation products such as ^{22}Na, ^{51}Cr, ^{54}Mn, ^{55}Fe, ^{60}Co, ^{63}Ni ^{65}Zn, ^{110}Ag, and ^{124}Sb to be detected in the environment [28, 29]. Stainless steel containing iron, nickel, and cobalt is an important material in nuclear power reactors and is used to construct nuclear test devices or their supporting structures [30, 31]. During neutron activation of the stable isotopes of cobalt, radioactive isotope ^{60}Co ($T = 5.27$ years) is produced. It is a beta emitter and decays into ^{60}Ni, with energy E_{max} of

0.314 MeV, after emitting two gamma quanta of energy, 1.173 MeV and 1.333 MeV [24]. Gamma spectrometry has been used to determine the precise activity of ^{60}Co in environmental samples [1]. The neutron activation of stable isotopes of iron produces a radionuclide of ^{55}Fe ($T = 2.685$ years), which decays by electron capture; it is a beta emitter of 6.9 keV energy [24]. The production of radioactive nickel isotopes through neutron activation yields ^{59}Ni, ^{63}Ni, and ^{65}Ni. The ^{63}Ni isotope is the most important of the three, because the activity ratio of ^{59}Ni/^{63}Ni is only 0.01. The ^{63}Ni isotope is a beta-particle emitter with a half-life of 100.1 years [32]. The analytical procedure for determination of ^{55}Fe and ^{63}Ni in environmental samples consists of the following stages:

(a) Co-precipitation of ^{55}Fe and ^{63}Ni in natural water with iron hydroxide
(b) Ashing of minerals (sediments and soils) and biological samples
(c) Separation of iron (^{55}Fe) with cupferron complex, and nickel (^{63}Ni) with dimethylglyoxime complex $(DMG)_2Ni$
(d) Purification of iron and nickel fraction by anion exchange resin (Dowex)
(e) Electrolysis of ^{55}Fe and ^{63}Ni on polished copper discs

The radiochemical yield is determined by the atomic absorption spectrometry (AAS) of stable Fe and Ni before and after electrodeposition. The activities of ^{55}Fe and ^{63}Ni are measured using an anticoincidence-shielded windowless low-level beta-particle gas-flow counter operating in the Geiger–Müller region. The counting gas consists of argon (99 %) and isobutene (1 %) [28, 33]. In the case of salts polluted with ^{55}Fe and ^{63}Ni, radiometric methods such as a scintillation counter in combination with high-resolution ion chromatography [34, 35] or adsorption of ^{63}Ni on Chelex 100 resin and purification by complexation with $(DMD)_2Ni$ [36] have been used. Also, acceleration mass spectrometry (AMS) has been used to determine ^{63}Ni in environmental samples [37].

15.2.3 Activity Measurement of ^{137}Cs

The artificial ^{137}Cs radionuclide is one of the most important long-lived ($T = 30.17$ years) fission products and a common contaminant. It emits β-radiation of two energies, 1176 keV (6 %) and 514 keV (94 %), exciting a 2.55 min isometric level of ^{137}Ba*. This isometric level de-excites itself by the emission of a single γ-ray of 661.66 keV [24]. In the equilibrium state, the activities of ^{137}Cs and ^{137}Ba* are the same. The ^{137}Cs activity can be determined directly using beta spectrometry or indirectly by measuring the ^{137}Ba* activity with gamma spectrometry. Direct determination of ^{137}Cs by beta spectrometry needs chemical mineralization of environmental samples and separation of cesium by adsorption on ammonium phosphomolybdate hydrate (AMP), followed by purification of ^{137}Cs from K and Rb on a cation exchange resin (Dowex 1), as well as co-precipitation of cesium hexachloroplatinate (Cs_2PtCl_6), and measurement of ^{137}Cs activity in a low-level flow beta counter [38]. Indirect methods involve drying and

homogenizing the analyzed samples and measuring the activity of $^{137}Cs(^{137}Ba^*)$ by gamma spectrometry. The direct methods are most accurate, but they are also labor-consuming and require calibration between the activity of ^{137}Cs and mass of Cs_2PtCl_6 sediment and mass of CsCl as a chemical tracer [1]. Calibration is usually carried out using ^{137}Cs standard solution of 1 kBq activity, 0.6–30 mg of CsCl carrier, and 50–200 mg of hexachloroplatinic acid (H_2PtCl_6). The calibration coefficient η between the mass of Cs_2PtCl_6 sediment and ^{137}Cs activity is calculated according to equation (15.2):

$$\eta = \frac{A}{\mathrm{CPM}_{100\%}} \tag{15.2}$$

where η is the calibration coefficient (Bq count^{-1} min^{-1}) (usual range 0.03–0.060), A is the activity of ^{137}Cs standard, and $\mathrm{CPM}_{100\%}$ is the number of counts calculated for 100 % cesium recovery.

The ^{137}Cs activity in the samples is calculated according to equation (15.3):

$$C = \frac{\mathrm{CPM}_\eta \cdot \eta \cdot 100\ \%}{m \cdot Y} \tag{15.3}$$

where C is the ^{137}Cs concentration (Bq g^{-1}), m is the mass of analyzed samples (g), η is the calibration coefficient (Bq count^{-1} min^{-1}), CPM_η is the number of counts during ^{137}Cs measurement in the beta counter (without background), and Y is recovery (%).

The standard deviation (SD) for the activity of ^{137}Cs is calculated according to equation (15.4):

$$\delta = \sqrt{\frac{\mathrm{CPM}_b}{t_p} + \frac{\mathrm{CPM}_t}{t_t}} \tag{15.4}$$

where CPM_b is the number of counts (including background) during sample measurement with the beta counter, CPM_t is the number of background counts in the beta counter, t_p is the sample counting time, and t_t is the background counting time.

15.2.4 Activity Measurement of ^{90}Sr

^{90}Sr is one of the most hazardous and dangerous radioactive isotopes. It is a pure beta emitter ($E_{max} = 546$ keV) and decays into another pure beta emitter, ^{90}Y ($E_{max} = 2283.9$ keV) [24].

Radiochemical methods for the determination of ^{90}Sr in environmental samples (water, soil, sediment, and biota) are based on the determination of activity of both

radionuclides in an equilibrium state or of only ^{90}Sr after its separation [5, 39]. The choice of analytical procedure depends on the kind and chemical composition of the samples, the time between data collection and analysis of samples, the type of radiometric measurement technique (scintillation, Cherenkov, or anticoincidence counters) as well as the method of separation (strontium or yttrium separation) [5]. The time between radiochemical contamination and sample collection is very important for ^{90}Sr analysis. According to Fischer [40], in fresh samples (apart from ^{90}Sr) the following radionuclides exist: ^{89}Sr, ^{91}Sr, ^{92}Sr, ^{91}Y, ^{92}Y, and ^{93}Y. Their activities are very small after 2–3 years; after this time, the activity ratio of the short-lived radionuclides in comparison to the activity of ^{90}Sr is less than 20. For fresh samples (up to 14 days old), rapid methods for the determination of ^{90}Sr are used, because ^{89}Sr as well as ^{90}Sr and ^{90}Y isotopes are also present in the analyzed samples. For analyzing older samples, accurate methods based on removing chemical and radiochemical pollutants are used [5].

The analytical methods for determining ^{90}Sr in environmental samples are separated into two groups, rapid and slow. Rapid procedures are used for determining ^{90}Sr in fresh soil and sediment samples collected a few days after contamination and are based on the sorption and subsequent strontium extraction on chromatography columns with different fillers (Sr-Spec$^{@}$, Amberlit resin), as well as ^{90}Sr activity determination in a liquid scintillation beta counter [41, 42]. Mateos and collaborators [43] proposed a sequence injection analysis to determine the ratio of $^{90}Sr/^{90}Y$ activity in mineral water samples 30 min after the separation of both radionuclides. Moreover, Friberg [40, 44] proposed the extraction of radionuclides from solid samples using concentrated HNO_3,subsequent separation of ^{90}Sr from ^{90}Y using HDEHP [acid bis (2-ethyloheksylo) phosphoric], and determination of ^{90}Sr activity in a Cherenkov counter. At 24 h after the separation of ^{90}Sr from ^{90}Y, the activity of ^{90}Sr in the organic phase constitutes 22 % of the initial activity of radiostrontium. For fresh samples, the total time of ^{90}Sr analysis is 50 days [40].

Slow methods provide the results of ^{90}Sr activity in environmental samples with minimum error, therefore the time of collection, preparation, and mineralization of samples is very important [39, 45]. In the cases of soil, sediment, bone, meat, and flora samples, incineration at a temperature of 450–600 °C for 4–24 h is recommended. In the separation process of ^{90}Sr mineralization by mixed HF, H_2SO_4, and HNO_3 acids [44, 46], and leaching by HNO_3 or HCl acids are used. Also lixiviation by NaOH, and HCl [39, 47], as well as 65 % HNO_3 [44, 48–50], or fuming HNO_3 are often used [39, 51, 52]. In these methods the measured activity of ^{85}Sr, added to samples as a tracer, in gamma spectrometry is used for calculating the analytical yield. Also, the determination of stable strontium by energy dispersive X-ray fluorescence spectroscopy (ED XRF) or AAS, before and after mineralization of samples, is used to calculate recovery [53, 54]. The analytical yield for soil samples is 2.7–58 % and depends on the content of strontium in the sample [53, 54].

In the case of trace analysis of ^{90}Sr (e.g., in natural water samples), carriers, most often ionic species such as Sr^{+2}, Ba^{+2}, Ca^{+2}, and Fe^{+3} should be used [39]. Ionic calcium is very similar to ionic strontium and should be removed from samples on the basis of differences between the solubility of calcium and strontium nitrates in

concentrated or fuming HNO_3. Chen and collaborators [55] proposed the separation of ionic calcium in the form of $Ca(OH)_2$ for samples containing more than 50 g Ca, where the ratio Ca/Sr is 250. Also, methods based on the extraction of ^{90}Sr using crown ethers [50, 52, 56] or separation in an ion-exchange resin are interesting [47, 50]. Many radionuclides, in particular Th, U, Ra, Ac, ^{210}Po, ^{212}Pb, and ^{214}Pb, contaminate samples and should be removed from the solution being analyzed. In the case of radium, co-precipitation of $Ba(Ra)Cl_2$ or $Ba(Ra)CrO_4$ sediments gives almost 100 % strontium separation from radium [55]. On the other hand, Th, U, Pa, Ra, and Ac in natural ores can be removed using the ion-exchange resin Dowex 1x8 or Dowex 50 Wx8 in solution after the separation of ^{90}Sr from ^{90}Y [57].

The most important stage of radiochemical procedure is separating ^{90}Sr from its decay product ^{90}Y. The methods used are based on co-precipitation with $SrSO_4$, SrC_2O_4, or $SrCO_3$ [39, 50–52, 55]; liquid–liquid extraction (e.g., using HDEHP) [44, 48, 53]; and liquid–solid extraction [41–43, 58–62].

Taking into consideration the method of ^{90}Sr analysis, the activity equilibrium state between ^{90}Sr and its decay product ^{90}Y is very important. This state is attained 12 days after the separation of radiostrontium [62]. The reliability of the received results of ^{90}Sr determination depends on the minimum detectable activity (MDA) [5]. The MDA should be calculated for each analysis sample. Generally, the separation of ^{90}Sr with the use of fuming HNO_3, and subsequent co-precipitation of radium, lead, and barium as chromates, is used for the analysis of flora, soil, ash filters, and water samples. The fusion products (e.g., ^{137}Cs) are removed by co-precipitation of the hydroxides, then transformed into yttrium oxalate, and the activity of ^{90}Y measured in a low-level proportional counter. The yield is controlled by measuring the activity of ^{85}Sr (gamma emitter) added to each sample before analysis as an internal tracer [1, 46]. The accuracy of the analytical results obtained should be verified in a validation process with the use of certified reference materials (CRMs).

After measurement using a liquid scintillation beta counter, the ^{90}Sr activity is calculated according to the equation (15.5) [1]:

$$A = \frac{29.55 \cdot N \cdot 100\ \%}{2 \cdot t \cdot \mathrm{eff} \cdot Y \cdot m} \tag{15.5}$$

where A is the ^{90}Sr activity (Bq g^{-1} dry wt.), N is the number of counts, t is the counting time (s), Y is the recovery of ^{85}Sr tracer (%) from gamma measurement, m is the mass sample (g), 29.55 is the proportionality factor between number of counts in ^{90}Y and ^{90}Sr–^{90}Y spectral energy, 2 is the value of the activity of either ^{90}Sr or ^{90}Y (these two activities are in equilibrium), and *eff* is the effective factor for beta radiation (usually from 0.90 to 1.00). It can be calculated according to equation (15.6):

$$\text{eff} = \frac{A_2}{A} \tag{15.6}$$

where A_2 is the ^{90}Y activity in the sample and A is the real ^{90}Y activity.

The detection limit (L_d) of ^{90}Sr is calculated [12] from equation (15.7):

$$L_d = 2.86 + 4.78\sqrt{(B + 1.36)} \tag{15.7}$$

where B is the number of background counts.

15.2.5 Activity Measurements of Natural and Artificial Alpha Radionuclides

15.2.5.1 ^{226}Ra Radium

^{226}Ra (half-life 1602 years) is a naturally occurring radioisotope of the ^{238}U decay series. Earth, marine and environmental scientists often require analysis of ^{226}Ra in natural samples, especially in waters, because of public health concerns and because it has proved to be a useful tracer of geochemical processes, particularly in the aquatic environment. The measurement of radium in natural, public, and drinking water supplies has become a matter of interest because it is one of the most hazardous elements with respect to internal radiation exposure [63–65]. Of environmental samples, natural waters are by far the most frequent sample matrices assayed for radium by liquid scintillation methods [64–66], the Cherenkov counting technique [67], alpha and gamma measurements, and inductively coupled plasma mass spectrometry (ICP-MS) [68]. Alpha spectrometry is the most sensitive technique, because it directly measures ^{226}Ra activity in a sample. Other techniques, especially liquid scintillation, the Cherenkov counter, and gamma spectrometry, are based on the measurement of ^{222}Rn as the ^{226}Ra decay product [69]. In natural water, radium is co-precipitated with manganese dioxide (MnO_2), but co-precipitated with microcrystal Ba(Ra)SO_4 sediment from minerals, sediments, and soil samples (after their mineralization). The activity of ^{226}Ra is measured using alpha spectrometry [70, 71]. The analytical recovery is calculated using ^{133}Ba (gamma emitter) or ^{224}Ra (alpha emitter) as internal tracer.

15.2.5.2 ^{222}Rn Radon

Radon is an inert noble gas and in the natural environment its most important isotopes are ^{222}Ra and ^{220}Rn. ^{222}Rn (half-life 3.8 days) is an immediate daughter nuclide of ^{226}Ra, but ^{220}Rn (half-life 55 s) is a product of ^{224}Ra decay [24]. From the radiological point of view, ^{222}Rn is the most important and measuring its activity in the air is very interesting [5]. The concentration of radon in air samples

can be measured immediately in ionizing or scintillation chambers, as well as by ^{222}Rn adsorption on active carbon [5]. The activity of ^{222}Rn decay products (as ^{214}Bi and ^{214}Pb in a radioactive equilibrium state) can be measured using gamma spectrometry or a scintillation counter (together with activity of ^{222}Rn). Other daughters such as ^{218}Po and ^{214}Po are adsorbed on glass filters and measured by passive implanted planar silicon (PIPS) detectors. Recently, methods have been developed based on adsorption of ^{222}Rn on active carbon and subsequent measurement of its alpha, beta, and gamma decay products [72, 73]. After 3 h, the activities of ^{214}Bi and ^{214}Pb are measured using gamma spectrometry, and they are proportional to the activity of ^{222}Rn on active carbon and in the air. This method needs the calibration of detectors for radon concentration in a radium chamber [73]. Another method is based on liquid scintillation, where the detector is a plastic cylindrical vessel of 30 mL volume (Pico-Rad) [5]. Inside this vessel there is active carbon for radon adsorption and silica gel for moisture adsorption. After 48 h of air exposition (95 % radon is adsorbed), a scintillation solution (based on xylene or toluene) is added to the vessel. Then, after 8 h, radon is transferred to the scintillation solution and the activity is measured in a liquid scintillation counter. In this method (Pico-Rad detector) the value of detection limits for the determination of ^{222}Rn is above 10 Bq m^{-3} with $\pm$ 10 % error [74].

Zikovsky and Roireau have developed simple methods for measuring radon in water using a proportional counter [75]. The method is based on purging radon from water with argon, which is bubbled through the water sample and then directed to the counting tube. The detection limit is 0.02 Bq L^{-1}, and this value compares favorably with other methods developed for determining radon in water, such as liquid scintillation, Cherenkov counting, or luminescence analysis [75–78].

15.2.5.3 Polonium (^{210}Po), Radiolead (^{210}Pb), Uranium (^{234}U, ^{235}U, ^{238}U), and Plutonium (^{238}Pu, $^{239+240}$Pu, ^{241}Pu)

A number of natural and artificial radionuclides are, or could be, used as indicators for studying geochemical and biological processes in the natural environment and their concentrations are very low.

Polonium is found in the natural environment, especially in uranium and thorium ores. Of seven natural radionuclides of polonium, ^{210}Po is the most important. It is an alpha emitter with energy of 5.305 MeV and half-life of 138.376 days [24]. Polonium is a very radiotoxic element and undergoes strong bioaccumulation in land and aquatic organisms [1].

Naturally occurring uranium contains three alpha-emitting radionuclides, ^{238}U (99.2745 %), ^{235}U (0.7200 %), and ^{234}U (0.0055 %). They are long-lived radioisotopes with half-lives of 2.455×10^5 years for ^{234}U, 7.037×10^8 years for ^{235}U, and 4.468×10^9 years for ^{238}U. The alpha-emitting energy for uranium isotopes lies between 4.040 and 4.776 MeV [24]. The activity of 1 Bq ^{238}U is equivalent to 81.6 μg total U in environmental samples. Moreover, the isotope ^{235}U undergoes a fission process and is used as nuclear fuel for nuclear energy [1].

Plutonium belongs to the group of artificial elements resulting from human activity and contains three alpha-emitting radionuclides, ^{238}Pu, ^{239}Pu, and ^{240}Pu, and beta ^{241}Pu isotope. These radionuclides are important from the radiological point of view as a result of their high radiotoxicity, long physical half-life, high chemical reactivity, and long residence in the environmental system. The half-life values are 87.7 years for ^{238}Pu, 2.411×10^9 years for ^{239}Pu, 6583 years for ^{240}Pu, and 13.2 years for ^{241}Pu [24]. The alpha-emitting energy of plutonium isotopes lies between 4.755 and 5.499 MeV [24]. The ^{239}Pu isotope is the most important, because it is fissile and is used to produce nuclear bombs. The principal source of plutonium in the environment (about 5 tons) is atmospheric fallout from nuclear weapon tests [1]. Radiochemical determination of plutonium isotopes in environmental samples is carried out using alpha spectrometry [79–81].

The radiochemical procedure for the simultaneous determination of natural (^{210}Po, ^{210}Pb, ^{234}U, ^{235}U, and ^{238}U) and artificial (^{238}Pu, $^{239+240}$Pu, and ^{241}Pu) isotopes in environmental samples is based on the following steps [1, 14]:

(a) Co-precipitation of radionuclides with manganese dioxide in natural water samples
(b) Mineralization of sediment, soil, and biota samples
(c) Sequential separation and purification of radionuclides on anion exchange resins
(d) Electrodeposition of polonium on silver discs, as well as electrolysis of uranium and plutonium on steel discs
(e) Measurement of activities of polonium (^{210}Po), uranium (^{234}U and ^{238}U), and plutonium (^{238}Pu, $^{239+240}$Pu, and ^{241}Pu) by alpha spectrometry

Before radiochemical analysis, recovery tracers are added to each sample (5–50 mBq of ^{209}Po, ^{232}U, and ^{242}Pu) [14]. Polonium in samples, after co-precipitation and mineralization, is spontaneously electrodeposited for 4 h at 90 °C in 0.5 HCl solution on a silver disc [82]. After polonium deposition, the solution is used to determine radiolead (^{210}Pb), uranium, and plutonium. The direct activity measurement of ^{210}Pb in natural samples is difficult, considering the low energy of emitted beta particles. The activity of ^{210}Pb is calculated indirectly by the measurement of ^{210}Po activity originating from the radioactive decay of ^{210}Pb [14]. Plutonium (IV) in concentrated acid solution (8 mol L^{-1} HNO_3 and 10 mol L^{-1} HCl) comprises the anion complexes $[Pu(NO_3)_6]^{2-}$ and $[PuCl_6]^{2-}$, which are adsorbed on an anion exchange resin (e.g., Dowex), whereas Pu(III) occurs as the Pu^{3+} cation [83]. Reduction of the adsorbed Pu(IV) anion complexes by ammonium iodide causes their decomposition to Pu(III). Apart from plutonium, neptunium, and thorium, the 8 mol L^{-1} nitric acid solution also contains the anion complexes $[Th(NO_3)_6]^{2-}$ and $[Np(NO_3)_6]^{2-}$. These complexes are adsorbed on an anion exchange resin but uranium (UO_2^{2+}), polonium (Po^{4+}), and iron (Fe^{3+}) pass through the column. On the other hand, in 10 mol L^{-1} HCl solution, uranium and iron exist as cations $UO_2Cl_4^{2-}$ and, $FeCl_4^-$, but thorium, americium, and actinium do not form anion complexes and are not adsorbed on an anion exchange resin. These

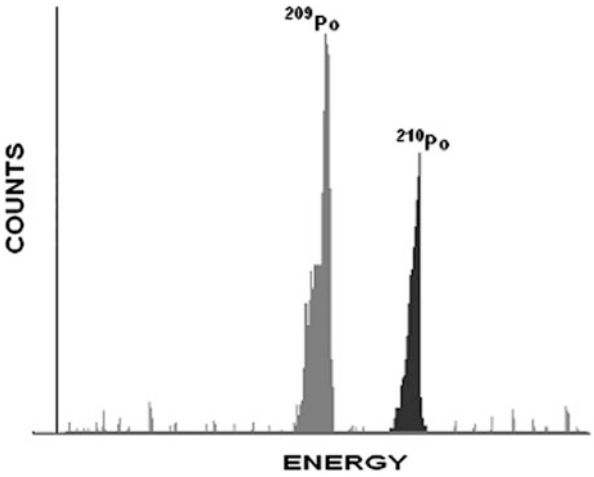

Fig. 15.3 Alpha spectra of polonium radionuclides (^{209}Po and ^{210}Po) [9]

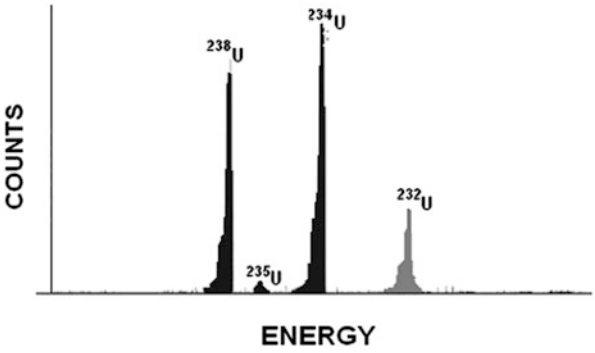

Fig. 15.4 Alpha spectra of uranium radionuclides (^{232}U, ^{234}U, ^{235}U, and ^{238}U) [9]

characteristics allow the purification of plutonium to remove contamination by other radioelements [1, 14].

Uranium U(IV) (also Fe, Co, Cu, Zn, and Cd) in 10 mol L^{-1} HCL solution is present in the form of the complex uranyl anion $UO_2Cl_4^{2-}$, which can be adsorbed on an anion exchange resin [84, 85]. The separation and purification of uranium from other elements is possible in sulfuric acid solution. When the H_2SO_4(aq) concentration is >0.01 mol L^{-1}, uranium exists in the anionic forms $UO_2(SO_4)_2^{2-}$ and $UO_2(SO_4)_3^{4-}$. In contrast to uranium, other elements (Fe, Co, Cu, Zn) do not form anionic complexes in sulfuric acid solution [1, 14].

After separation and purification, the pure fractions of uranium and plutonium are electroplated on polished stainless discs and the activities of their radionuclides measured using alpha spectrometry. The distribution value of alpha detectors, which is between 17 and 20 kev, is very important. Two radionuclides (^{239}Pu and ^{240}Pu) are measured together because the difference between the energy of their alpha particles is less than 15 keV [1, 14]. Figures 15.3, 15.4, and 15.5 present typical spectra for the alpha measurement of polonium, uranium, and plutonium [1].

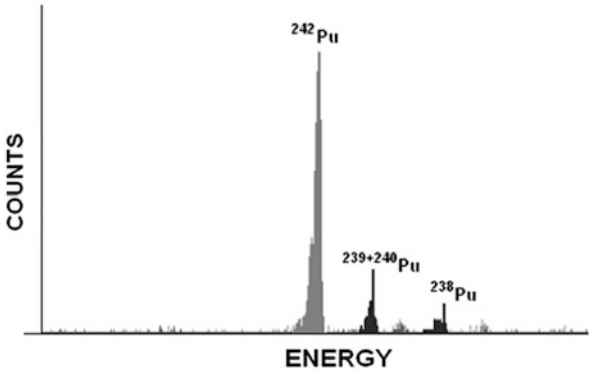

Fig. 15.5 Spectra of plutonium radionuclides (^{238}Pu, $^{239+240}$Pu and ^{242}Pu) [9]

15.2.5.4 Calculation of Polonium, Radiolead, Uranium, and Plutonium Radionuclides

The activities of ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, and $^{239+240}$Pu are calculated [1, 14] using equation (15.8):

$$A_i = \frac{I_i}{e_i \cdot Y_i} \pm SD_i \tag{15.8}$$

where i is ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, or $^{239+240}$Pu; A_i is the activity (Bq); I_i is the count rate of the sample (without background) defined as the ratio N_i/t_p; N_i is the ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, or $^{239+240}$Pu count (without background); t_p is ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, or $^{239+240}$Pu counting time (s); e_i is detector efficiency; Y_i is the recovery of ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, or $^{239+240}$Pu; and SD_i is the standard deviation (uncertainty) of measured sample activity.

The recovery of ^{210}Po, ^{234}U, ^{235}U, ^{238}U, ^{238}Pu, and $^{239+240}$Pu are calculated [1, 14] according to equation (15.9):

$$Y_i = \frac{I_i}{e_i \cdot s} \tag{15.9}$$

where Y_i is the recovery, I_i is the count rate of recovery tracers (^{209}Po, ^{232}U, and ^{242}Pu), s is the ^{209}Po, ^{232}U, and ^{242} Pu activity added before radiochemical analysis (Bq), and e_i is the detector efficiency.

The SD for the activity of polonium, uranium, and plutonium is calculated according to [1, 14] equation (15.10):

$$SD = \frac{A_0}{A_t} \cdot \frac{\sqrt{\frac{I_p}{t_p} + \frac{I_t}{t_t}}}{Y \cdot e} \tag{15.10}$$

where A_0 is the activity of ^{210}Po at the time of electrodeposition (Bq), A_t is the activity of ^{210}Po at the time of counting (Bq), I_p is the sample count rate (with background), I_t is the background count rate, t_p is the sample counting time (s), t_t is the background counting time (s), Y is the recovery, and e is the detector efficiency.

If the background count is very low, and during the analysis time $A_0 = A_t$ (for uranium and plutonium), then the SD is calculated according to [1, 14] equation (15.11):

$$SD = \frac{\sqrt{N_p}}{Y \cdot e \cdot t_p} \tag{15.11}$$

where N_p is the number of counts of uranium (^{234}U, ^{235}U, and ^{238}U).

The activity of ^{210}Po in environmental samples is calculated on the basis of the electrodeposition time of polonium on a silver disc using [1, 14] formula (15.12):

$$A_0 = A_t \cdot \exp\left[\frac{t \cdot \ln 2}{T}\right] = A_t \cdot \exp(0.00502 \cdot t) \tag{15.12}$$

where A_0 is the ^{210}Po activity at the time of electrodeposition on a silver disc (Bq), A_t is the ^{210}Po activity at the time of counting (Bq), t is the time interval between electrodeposition and the ^{210}Po count (days), and T is the ^{210}Po half-life (138.4 days).

The radioactivity of radiolead is measured by an indirect method, where ^{210}Pb activity is estimated on the basis of ^{210}Po in growth after the lead fraction has been purified and stored for several months (up to 2 years). The ^{210}Pb activity at the time of sample collection is calculated [1, 14] according to equation (15.13):

$$A_0\left(^{210}\mathrm{Pb}\right) = \left[\frac{A_2\left(^{210}\mathrm{Po}\right)}{1 - \exp[-k(t_2 - t_1)]}\right] \tag{15.13}$$

where $A_0(^{210}$Pb) is the activity of ^{210}Pb at the time of sample collection, $A_2(^{210}$Po) is the activity of ^{210}Po originating from ^{210}Pb decay following the second electrodeposition, t_1 is the time between collection and the first ^{210}Po count, t_2 is the time between the collection and the second ^{210}Po count, and k is the ^{210}Po decay constant.

The impact of the Chernobyl plutonium fraction in environmental samples is calculated [1, 14] using the following equation (15.14):

$$F_{ch} = \frac{R_{obs} - R_n}{R_{ch} - R_n} = \frac{R_{ch} - 0.04}{0.56} \quad (15.14)$$

where R_{obs} is the $^{238}Pu/^{239+240}Pu$ activity ratio in the sample analyzed, R_n is the $^{238}Pu/^{239+240}Pu$ activity ratio in the global atmospheric fallout (0.04), and R_{ch} is the $^{238}Pu/^{239+240}Pu$ activity ratio in the Chernobyl accident (0.60).

15.2.6 Activity Determination of ^{241}Pu

^{241}Pu is a low-energy emitter with E_{max} of 21 keV and a half-life 13.2 years. ^{241}Pu can be determined directly by measurement in a beta proportional counter and in a liquid scintillation counter (samples with a relatively high content of ^{241}Pu) [86, 87], and indirectly by alpha spectrometric measurement of its daughter radionuclide ^{241}Am [88, 89].

The measurement is based on the growth of ^{241}Am and can be carried out only after a long growth period, between 4 and 20 years. Even after 4 years, the activity ratio of $^{241}Am/^{241}Pu$ is only 1:166. Thus, the lower limit of detection for ^{241}Pu by direct measurement using a proportional counter is about 10 mBq, whereas ^{241}Am build-up requires about 200 mBq for detection [90]. The plutonium alpha spectra obtained have to be compared with the same spectra obtained 4–20 years earlier. This then enables the ^{241}Pu content to be estimated on the basis of the increase in the 5.49 MeV peak of ^{241}Am, which takes into account the ^{238}Pu present in the environmental sample from the Chernobyl accident. The ^{241}Pu activity is calculated [88] according to equation (15.15):

$$A_{Pu_0} = 31.11 \cdot \frac{A_{241_{Am}} \cdot e^{\lambda_{Am} \times t}}{(1 - e^{-\lambda_{Pu} \cdot t})} \quad (15.15)$$

where A_{Pu_0} is the ^{241}Pu activity at the time of sampling, $A_{241}{}_{A}m$ is the ^{241}Am activity measured after 4–20 years, λ_{Pu} is the decay content of ^{241}Pu (0.050217 year^{-1}), λ_{Am} is the decay content of ^{241}Am (0.001604 year^{-1}), 31.11 is the $\lambda_{Pu}/\lambda_{Am}$ ratio, and t is the time from sampling to the measurement of ^{241}Am (4–20 years).

15.2.6.1 Estimation of Errors in Radioanalytical Methods

The errors inherent in measuring the activity of polonium, uranium, and plutonium can be assessed by determining radionuclides in CRMs in international laboratory exercises and using the CRM produced by the International Atomic Energy Agency (IAEA). The accuracy was estimated to be less then 9 % for ^{210}Po; 7.5 % for ^{234}U, ^{235}U, and ^{238}U; and 6 % for $^{239+240}Pu$, but precision was estimated to be below 10 %. The chemical recovery was 95–99 % for ^{210}Po, 85–95 % for ^{238}U, and 50–

85 % for $^{239+240}$Pu [1, 14]. The MDA value during the measurement in alpha spectrometry was calculated on the basis of equation (15.16):

$$MDA = 2.71 + 4.65\sqrt{B/t}\eta Y \tag{15.16}$$

where B is the number of background counts, t is the counting time, η is the counting efficiency, and Y is the recovery of alpha measurement [5].

For 3–10 day measurements, the MDA for analyzed ^{210}Po, ^{238}U, and $^{239+240}$Pu radionuclides was 0.08–0.15 mBq [1, 14].

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