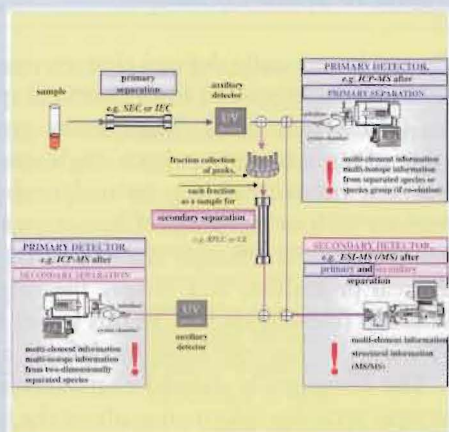CHAPTER 25

SPECIATION OF TRACE ELEMENTS

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CONTENTS

I. INTRODUCTION

The determination of trace elements has assumed a place of prominence in the life sciences. Elements present at even minimal concentrations in biological and environmental matrices can have a significant influence on vital functions, depending on the amount present. The study of, for example, pathophysiological processes in the human body requires the determination of elements at concentrations measured in $\mu g\,L^{-1}$, $ng\,g^{-1}$, and even $pg\,g^{-1}$. The higher concomitant amounts of organic and inorganic components make it difficult to determine the presence of trace elements. Moreover, it is a complex process that progresses from an initial trace element analysis to the final statement of

biological implications, one that requires close collaboration between the analytical chemist and life scientist. Furthermore, it should be kept in mind that the concept of *zero tolerance* for potentially toxic elements has been replaced by the more scientific notions of *safe ranges of exposure* and *range of safe intake*.

Over the last two decades, analytical chemists have come to realize that, in general, the total concentrations of chemical elements cannot provide information about their mobility, bioavailability, and eventual impact on ecological systems and biological organisms. Only knowledge of the chemical species of an element can provide information regarding possible chemical and biochemical reactions and thus lead to a greater understanding of toxicity or essentiality. It is also worth stressing, in this context, that new trace elements are continually being added to the list of those that are known or suspected to be essential.

Until now, already established separation and detection methods had to be combined in novel ways and modified according to particular speciation problems. Combination and hyphenation of separation techniques and element- or molecule-selective detection systems are generally the approaches of choice for speciation analysis; however, further methodological developments are still necessary, primarily for hyphenation and quality-control strategies. Investigations on quality control have shown that changes in the original species information can easily occur during sampling, sample

preparation and storage, separation, and detection. For specific details on various analytical techniques, see Chapter 29, this volume.

A. Definitions of Terms Related to Speciation

The use of concepts and terms related to chemical speciation in recent years still reflects a certain degree of inconsistency within the scientific community. In recognition of the importance of standard terminology from the viewpoint of both interdisciplinary communication and constructive interaction with decision makers, the International Union of Pure and Applied Chemistry (IUPAC) has undertaken a collaborative effort of three of its divisions and reached a consensus on some basic definitions that can be used by specialists in the discipline of speciation (Templeton et al., 2000). One of the major conclusions of this working group was that the term "speciation" should be restricted to the distribution of an element among well-defined chemical forms. A clear distinction was also made between *speciation* and *fractionation*. As it is quite crucial for those working in this field to fully abide by such definitions, they are reported below verbatim:

- *Chemical species* (of a chemical element)—Specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.
- *Speciation analysis* (in analytical chemistry)— Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.
- *Speciation of an element*—Distribution of an element among defined chemical species in a system.
- *Fractionation*—Process of classification of an analyte or a group of analytes from a certain sample according to physical (*e.g.*, size, solubility) or chemical (*e.g.*, bonding, reactivity) properties.

The analytical activity of identifying and measuring species includes a clear identification of the species (elements and possibly the binding partners) and exact quantification in representative samples, as well as quality control (Caroli, 1996; Michalke, 1999a). If the identification and quantification of a chemical species cannot be performed, the analytical procedure can only lead to operationally defined species characterization.

B. Operationally and Functionally Defined Characterization of Species (Groups)

Operationally and functionally defined characterization of species has to be distinguished from chemical speciation analysis (Ure et al., 1993). The former gives a characterization of molecule groups (not single species) that show a similar behavior during an analytical procedure (operation) such as extraction. Characterization of the molecule groups is strongly dependent on the selected analytical procedure, and usually the original species information (identity) is lost. In this sense, fractionation is regarded as an operationally defined characterization. The functionally defined characterization of species (groups) provides information about the function of species groups (not singly identified species) in organisms and their impact on living systems (Caroli, 1996; Mota & Simaes Gonçalves, 1996). Neither is considered to be a real chemical speciation analysis, as identification of a single species does not actually take place.

C. Necessity and Reasons for Element Speciation

The quality and quantity of the relevant element species in a matrix, rather than the total element concentration, are greatly responsible for the mobility, bioavailability, and ecotoxicological or toxicological impact of an element (Florence, 1989; Templeton et al., 2000). Elements usually interact as parts of macromolecules (*e.g.*, proteins, enzymes, hormones) or according to their oxidation state; therefore, only current knowledge about a species provides a reason for further assessing whether or not it is toxic, without (known) impact at a specific concentration, or essential. Problem-related speciation analysis appears to play a key role in effectively assessing the risk posed by elements in the environment. Also, from a health viewpoint, the consequences of a trace element's essentiality, depletion, or toxicity can be better determined, and the development of diagnoses and potential therapies is improved. Some examples are provided in the following text to illustrate how different species of a given element can have different impacts (see Table I). It must be realized that, in speciation analysis, the analytical procedure generally interacts with the separation and detection of a species. These interactions usually shift equilibria among the species and possibly change some of the species themselves. The nature and extent of these alterations, as well as a critical discussion of the results achieved, should therefore be an essential part of well-conducted speciation analysis.

TABLE I. Impact of Various Species of Elements

Element	Impact of Species
Arsenic	As(III) and As(V) are toxic; arsenobetaine is nontoxic.
Chromium	Cr(III) is essential; Cr(VI) is highly toxic and cancer-promoting.
Copper	Ionic Cu(II) is toxic in aquatic systems; humic complexes of Cu are almost nontoxic.
Iodine	Thyroid hormones influence an extended range of biochemical reactions in organisms and play an active role in immune defense. Triiodothyronine (T_3) shows about a fivefold effectivity compared to thyroxin (T_4), but it comprises only about 20% of the total iodine hormones.
Iron	Absorption capacity for Fe(II) is lower compared to Fe(III), but only Fe(II) is effective against Fe deficiency. This is important for supplementation; however, Fe(III) is utilized efficiently following reduction by ceruloplasmin.
Mercury	Inorganic Hg salts are less dangerous than methylated forms; these are more toxic and can be enriched (e.g., up to 10,000-fold in fish).
Platinum	Pt(0) is nearly completely insoluble in water. After emission from car catalysts as Pt(0), Pt species transformation occurs, and solubility in water as well as its availability are significantly increased.

Source: Data from Florence (1989) and Lustig *et al.* (1998).

D. Useful Fields for Speciation

Speciation is particularly relevant to the environmental field, as well as to biology and medicine. Food chemistry and nutrition, in turn, can also greatly benefit from the speciation approach, which can act as an interface between these two fields. The nature and amount of manmade species are altering natural species formation and equilibria; consequently, trace element mobility and bioavailability may be influenced and modified. Bioavailability is directly linked to biochemical mechanisms within the organism. Thus, the fate of a species—such as adsorption to membranes, transport and incorporation into larger molecules (*e.g.*, enzymes), and enrichment or excretion—may be modified and result in an unbroken path from environmentally changed species to toxicity, deficiency, or growth in biological systems.

E. Species Impact and Mechanism in Biological Systems

A necessary prerequisite for an elemental species to interact with an organism is that the species must be able to cross the cell membrane and participate in biochemical paths and reactions (Morrison, 1989). Several intake mechanisms are known, as detailed in Table II. These uptake mechanisms result in an enrichment of element species in the organism by a factor of 10^2 to

10^5. In some cases, toxic concentrations are reached even when the original species concentration in the environment is low (Morrison, 1989). The uptake and subsequent metabolization of element species is obviously dependent on the nature of the species itself, as are the consequences of that uptake and metabolization:

- *Immediate excretion without any interaction*—This action is considered beneficial for species having toxic potential and adverse for essential element species.
- *Interaction with the organism and participation in metabolic paths*—This result is considered to be beneficial for essential element species and adverse when toxification and a reduction in enzymatic selectivity and turnover rate occur. The replacement of an essential element by another one in the reactive center of the enzyme can sometimes cause enzyme damage.
- *Intracellular toxicity*—Such toxicity often appears when intracellular species transformations occur. The displacement of essential elements in the reactive enzyme centers results in inactive enzyme–metal complexes (*i.e.*, new species). Conversely, metal exchange at a protein can also be a detoxification reaction (*e.g.*, via metallothioneins [MTs]).
- *Metallothionein transcription*—Metallothioneins, 7- to 10-kDa proteins with about 30% S amino acids (~30% cystein) have a high affinity to metals such as cadmium, copper, mercury, and zinc. In

TABLE II.   Cell-Entering Mechanisms for Elemental Species

Diffusion	Diffusion is dependent on the size and lipophilic nature of the element species. It is fast and efficient for lipophilic molecules and is associated with high toxicity. Ionophores, which have such an increased lipophilic nature, may form complexes with (lipophobic) metals. These "excluded metals" are then transported across the cell membrane by the ionophores. In the cell, the metals are set free again and recomplexed by proteins or other ligands.
Active transport by ATPase-driven ion pumps	The ATPase-driven uptake mechanism has been proven for some essential elements such as Cu^{2+}, Zn^{2+}, and Ni^{2+}.
Carrier/shuttle transport	This transport mechanism is typically shown by proteins and hormones.
Uptake via ion-selective channels/active transport by electrical potential	Ion-selective channels have been tested for cations such as Ca^{2+} and K^+. Transportation across the membrane is dependent on the D_μ^{H+}-membrane potential. A potential higher than $-70\,mV$ opens voltage-gated channels.

Source: Morrison, G. M. P., In *Trace Element Speciation: Analytical Methods and Problems*, G. E. Batley, Ed., CRC Press, Boca Raton, FL, 1989, pp. 25–42. With permission.

organisms experiencing such a metal load, a genetic MT transcription is induced that increases the concentration of the "offered" ligand to the toxic metals. The generated MT-metal complex is excreted via kidneys, thus protecting the reactive centers of enzymes.

• *Availability of many elements*—Elements must be available for the organism as well-defined species and at suitable quantities to guarantee a normal health status. A good example of this is chromium, which is essential in the trivalent oxidation state and highly toxic and carcinogenic in the hexavalent oxidation state.

F. Reference Values and Ranges

The achievement of reference concentration values and intervals for elements in biological and environmental matrices is of paramount importance in the detection of imbalances that can adversely affect human health and ecosystems. An exhaustive overview of problems and applications related to this issue was published several years ago (Caroli *et al.*, 1994). In this context, "normal values" are provided as tolerance limits for those elements that may be undetectable in human organs. Elements essential for life, on the other hand, are homeostatically regulated, and their concentrations are expected to fluctuate within narrow limits for each species under normal conditions. Although doing so is still far from feasible, there is no doubt that for chemical speciation determining reference figures will

become even more crucial than obtaining knowledge regarding the total amount of a particular element.

II. SPECIATION ANALYSIS: SAMPLING, STORAGE, PREPARATION, SEPARATION, DETECTION

The direct determination of trace elements in samples is an important problem in technology, industry, and research, because decomposition and preconcentration procedures, as well as the storage of trace analytes in solutions, are often sources of concern. The accuracy of analytical results can, in fact, be threatened by these pretreatments. Only a few methods exist for direct analytical determinations in solids. In many cases, the detection power and reproducibility of spectral analyses are inadequate to meet the needs of analysts. This less-than-ideal performance is of particular concern in speciation analysis, which requires a series of carefully planned steps among which chemical and/or physical pretreatments of the material being tested are almost always mandatory. In this context, sampling and sample preparation are of prime importance. Without proper sampling and sample treatment procedures, there is little chance that any speciation analysis will be able to provide reliable data upon which human health or environmental decisions can be safely based. Thiers (1957) stated that, unless the complete history of samples is known with certainty, the analyst is well advised not to

spend time analyzing them. The container in which the sample is stored is itself a potential source of contamination, as is the sample pretreatment procedure or manipulation or the analyst. Volatilization is another source of error. If unexpected changes to the form of the element occur, such as oxidation state, extent of chelation, or organometallic state, then clearly the species has been changed, and the original species identity and amount cannot be ascertained, thus defeating the purpose of the experiment. On the other hand, highly reliable data can be obtained with careful evaluation of the potential chemical changes in the sampling and sample preparation process.

A. Sampling

The sampling step is all the more critical in speciation analysis and usually shows uncontrolled and irreversible interferences in the species equilibria (Dunemann & Begerow, 1995). Sampling should be designed to preserve the original information about native species; however, existing techniques are often inadequate for the problem at hand. They must be adapted to the actual situation with regard to the element species of interest in the given matrix (Kersten et al., 1989). Several problems have been identified; for example, wall adsorption effects have been described, as well as contamination from the sampler and alteration of biological or chemical equilibria or oxidation by atmospheric oxygen (Caroli, 1996). When sampling natural water, unintended contamination of the probe frequently occurs in the surface microlayer, which is usually enriched by trace metal species. This contamination can be avoided by taking water samples 0.5 to 1.0 m below the surface. Also, materials such as dust and airborne particulate matter may be available in only very limited amounts (0.02–0.05 g); therefore, their analysis requires the use of methods with high detection power.

In biological samples, contamination from syringes, metal scalpels, and other metal tools can alter the species pattern of other elements. Species alterations due to bacterial activity have also been observed (Dunemann & Begerow, 1995). The extent to which the sample is representative of a chemical species is often not confirmed. Each of the thousands of possible major biological and environmental materials suffers from different matrix effects; even the urine of a given individual can differ substantially in its concentration of salts from one day to another. An overview of the sampling problems encountered under critical conditions in an extreme environment has recently been published (Caroli et al., 2000).

B. Sample Storage and Processing

Samples typically cannot be analyzed on site; therefore, storage becomes necessary and should be as short as possible. For replicate measurements on a given sample and for many applications, longer storage times become necessary. Proven storage techniques typically used for trace element analysis may be inappropriate for element speciation studies; for example, acidification cannot be used for element speciation studies because pH changes affect species composition and thus alter native speciation information.

Unreliable data can be found in the analytical literature due to the ubiquitous nature of certain elements and unaware analysts. Such elements are present in gloves, rubber stoppers, and anticoagulants. Among the many potential sources of contamination are dust, dirt, cosmetics, disinfectants, talc and dust on gloves, and metallic corrosion products. Laboratory dust can contain up to 0.3% aluminum, 0.3% calcium, 0.3% iron, 0.8% potassium, 0.2% magnesium, 0.3% sodium, 0.2% lead, 2% sulfur, and 0.2% zinc. Preservatives are also rich in elements; heparin contains calcium and zinc, and formalin contains iron, manganese, and zinc. These contaminants are likely to disturb not only trace element analysis but also element speciation. Such contaminations shift species pattern and may rearrange complexes in a sample. Even when the element species of interest is not the contaminating element, a shifting of species equilibria or changes in complexes are possible. Drying of samples can result in the loss of element species (predominantly volatile species). Problems of subsampling must also be considered. In most regulations and directives, analytical methods and sampling are only briefly addressed. Sampling and analysis should be described as accurately as possible to profit from the excellent spectrometric methods available.

Samples should not be contaminated or destabilized during storage and sample handling. Clean-room conditions and precleaned vials, among other precautions, must be used throughout. Sample preparation should be as simple as possible to reduce possible alterations. For this reason, storage time should be kept to a minimum, preferably at 4°C. For long-term storage, freeze-drying or shock freezing at −80°C is recommended; however, when the freeze-drying procedure is used, the process should allow for control of the sample temperature.

Even then, volatile species may be lost. No acid additions or other pH-changing agents are allowed, nor are repetitive, slow thaw/freeze cycles. Glassware can have ion-exchange properties; thus it is less suitable than polymer materials (Urasa, 1996). Before storing water samples, particulate matter must be removed to avoid species condensation and a shift in elemental speciation. On the other hand, colloids must not be removed, as they are considered to be a species group or fraction themselves. Mercury is a well-known example of the problems that can be encountered. The possibility of random errors in the determination of mercury due to migration phenomena is primarily associated with the sampling, pretreatment, and storage of samples. Mercury concentrations in water samples stored for a long time are strongly affected by several physical phenomena (*e.g.*, sorption and desorption), dissolution, and passage through the walls of the container. The errors due to these processes are, however, in most cases independent of the analytical method used.

For biological samples, storage and sample preparation should be as short as possible, preferably at 4°C. Recently, solid biological samples were investigated for enzymatic extraction employing protease (different types) or lysozyme. Also, hotwater extractions were compared to diluted HCl leaching. When yeast was used for sample extraction, the efficiencies were quite high (*e.g.*, reaching, 80–100% of the total Se content of the sample after a protease treatment) (Potin-Gautier *et al.*, 1997). HCl leaching was used successfully for mimicking gastric juice digestion (Crews *et al.*, 1996), but it was found to be less suitable for speciation in a bacterial sample (Michalke *et al.*, 2002). Species stability in soils and sediments during storage can be a problem, as volatile species may be lost. Generally, extraction procedures are required for further analysis. This can obviously cause species alteration, depending on the chosen extractants, and bring subsequent analysis into the field of "operationally defined" approaches. As Pickering (1981) wrote: "The gained results often are bound to errors and limitations, finally leading to wrong or misunderstanding data."

Usually, extractions are single extractions aimed at availability studies or sequential extractions. Single extractions often use leaching agents such as H_2O, $NaNO_3$, NH_4NO_3, KNO_3, $CaCl_2$, CH_3COONH_4, EDTA, or CH_3COOH. A promising single extraction method recently was described for tin (Sn) species using low-power, short-time, microwave-assisted leaching of soil or sediment. Here, CH_3COOH or diluted HNO_3 were used as extractants, and microwaves were applied at only 30 W at approximately 60°C for a few minutes.

TABLE III. Example of Steps in Sequential Extraction

Steps	Remarks
1. Exchangeable, adsorbed	—
2. Carbonate fraction	Subsequent fractions are usually not interfered.
3. Reducible fraction	This step is usually accomplished by using hydroxylamine–HCl/acetic acid; however, selectivity is doubtful.
4. Oxidizable fraction	Possible binding mechanisms are adsorption, chelating, and complexation; differentiation from step 3 is often impossible.
5. Residual fraction	For example, aqua-regia.

The stability of organotin was preserved, and extraction efficiency approached 100% (Rodriguez-Pereiro *et al.*, 1997).

Sequential extraction procedures attack the sample by utilizing consecutive leaching agents of increasing strength and ability to interact with the sample matrix. Typical steps are summarized in Table III. Representative extraction procedures have been described in a number of papers (Zeien & Bruemmer, 1989; Ure *et al.*, 1993). These procedures try to leach the elements from the different compounds and complexes in a stepwise fashion. Usually, however, the selectivity is not high enough. Problems may arise because of the pH dependence of the extraction step. Sample matrices can also alter pH and therefore selectivity.

C. Speciation Approaches: Direct Speciation Methods or Combined (Hyphenated) Techniques

After sampling, storage, and sample preparation, species are identified and analyzed. Direct speciation approaches can provide full information about the species in a sample without any additional (separation) method; that is, they can directly quantify the species. Such methods include nuclear magnetic resonance (NMR) or (in special cases) anodic stripping voltammetry (ASV) and cathodic stripping voltammetry (CSV). The concentration ranges detectable by NMR, however, are far too high for real-world samples of biological or ecological relevance. With ASV and CSV,
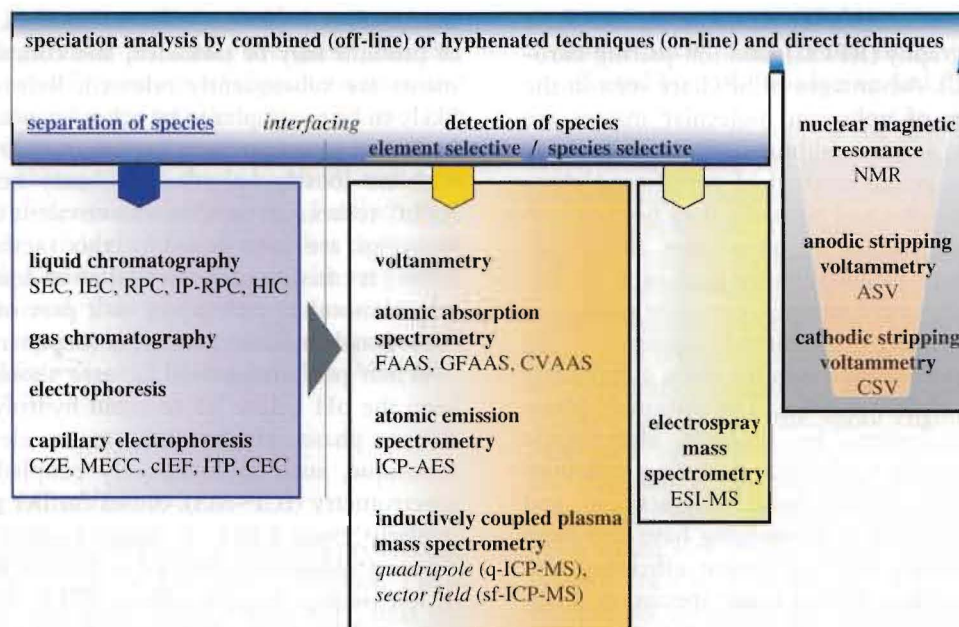
speciation analysis by combined (off-line) or hyphenated techniques (on-line) and direct techniques			
separation of species *interfacing*		detection of species element selective / species selective	nuclear magnetic resonance NMR

liquid chromatography
SEC, IEC, RPC, IP-RPC, HIC

gas chromatography

electrophoresis

capillary electrophoresis
CZE, MECC, cIEF, ITP, CEC

voltammetry

atomic absorption spectrometry
FAAS, GFAAS, CVAAS

atomic emission spectrometry
ICP-AES

inductively coupled plasma mass spectrometry
quadrupole (q-ICP-MS),
sector field (sf-ICP-MS)

electrospray mass spectrometry
ESI-MS

anodic stripping voltammetry
ASV

cathodic stripping voltammetry
CSV

FIGURE 1 Two speciation approaches are shown: one using direct methods, the other employing coupled techniques that include separation, interfacing/coupling, and selective detection. The latter are commonly used for element speciation due to flexibility and sensitivity.

quantification is rarely possible in samples with high organic loads.

The most usual approach for element speciation is based on combined (or hyphenated) systems. Here, species are selectively separated and then the elements in the various chemical forms are selectively detected. For increased quality control, molecule-selective detection is coupled to separation devices. These combinations provide extended flexibility and a broad applicability. Disadvantages include the increased complexity and thus increased risk for failure of the systems. Also, species equilibria can be drastically altered during separation due to dilution, and some components can be removed from the chemical equilibrium. Species transformation and destruction are likely consequences; therefore, the total separation time should be short compared to the transformation rate of species. Figure 1 gives an overview of the various speciation methods. In the following section, separation mechanisms are described and their features are taken into account from the viewpoint of element speciation. The hyphenation of separation techniques to element detectors is also discussed.

1. Liquid Chromatography

One of the most important advantages of liquid chromatography (LC) is the ample selection of separation

mechanisms and the use of various mobile and stationary phases which provide nearly all the necessary techniques for separation of element species; therefore, specific problem-related speciation analysis is often possible that meets the requirements for species stability and efficient separation. Preservation of original species information is at least as necessary as good separation and influences the choice of separation mechanisms and reagents. Many stationary phases and buffers or organic modifiers can denature native species. Chelating eluents or ion exchangers may cause recomplexation of free or labile bound metal species (Dunemann & Begerow, 1995). A more general disadvantage is seen in the existence of a stationary phase, as compared, for example, to capillary electrophoresis (CE); the large surface allows adsorption effects, contamination, or miscellaneous alterations of the species to occur (Harms & Schwedt, 1994).

The mobile phases, too, can cause severe alterations of species, even if they may be very effective in separation. Although doubly distilled water is highly pure, some denaturing effects on biomolecules have been observed. Buffers can stabilize biomolecules, but they may also alter species equilibria. Complexing tendencies or input from metal contamination may also occur (Arnaud et al., 1992).

The commonly used LC separation techniques in speciation are size exclusion chromatography (SEC),

ion-exchange chromatography (IEC), reversed-phase liquid chromatography (RPLC), and ion-pairing chromatography (IPC). Advantages of SEC are seen in the fact that samples of unknown molecular masses are characterized in a mass-calibrated chromatographic system. SEC is a gentle method of performing chromatographic separation and normally does not result in a loss of element species or on-column alterations, although the column exhibits limited peak capacity. For complex multicomponent samples, complete resolution of the peaks is normally not achieved. Szpunar (2000b) reported shifting retention times for some compounds beyond the final elution volume. The stationary phase is not totally uncharged; for example, electrostatic effects have been observed when analyzing cadmium species. Adsorption, hydrophobic interactions, and species-specific affinities or H-bridging have also been observed. IEC shows high separation efficiency and wide applicability, thus solving many speciation problems. The relative retention of the ionic species is determined by three variables: namely, pH, ionic strength of the mobile phase, and nature of the ion exchanger. On the other hand, hydrophobic interactions between the sample and the non-ionic carbon backbone of the stationary phase cause organic ions to be retained in a way typical for RPLC (Mikes, 1988). The pore size of the resin particles is an important parameter for the success of the separation. Often, loosely bound metal ions are lost or replaced by other metals originating from the buffer (Quevauviller *et al.*, 1996); thus, IEC is a good fit for the separation of covalent bound element species in different valence states, such as Cr(III)/Cr(VI) or Sb(III)/Sb(V)/methyl-Sb (Lintschinger *et al.*, 1998). Another frequent application is for covalent bound Se amino acids (Michalke *et al.*, 2002) or a high number of arsenicals (Gössler *et al.*, 1997).

The predominant advantage of RPLC is the wide analytical spectrum available; this very effective separation technique provides high resolution of species, and the flexibility offered by the multiple mobile phases allows the addition of ion-pairing reagents for analysis of ionized molecules. Obtaining results is usually easy and fast. The normally high reproducibility is a significant advantage in speciation analysis (Mikes, 1988). In practice, the stationary phase can exhibit ion-exchange properties or undesired adsorption effects, especially for basic analytes. At pH values higher than 4, these basic analytes can be adsorbed tightly. Usually, two different eluents are necessary, at least one of them containing a considerable amount of organic modifier. The polar eluent often shows high complexing tendencies. Organic solvents and acids may easily change element

species such as protein–metal complexes. The structure of proteins may be unfolded, and complex-bound elements are subsequently released. Released metals are likely to be recomplexed by other ligands, which results in species transfer reactions; hence, only those analytes with no loosely bound metals may be separated by RPLC technique. Species with covalent element-ligand bondings are best suited to this method; therefore, RPLC is often used in parallel or in combination with other smoother techniques as a part of the so-called orthogonal speciation concepts (Szpunar, 2000a).

When performing RPLC, care should be taken to keep the pH below 7.5 to avoid hydrolysis of the stationary phase. Hyphenation to an element-selective technique, such as inductively coupled plasma–mass spectrometry (ICP–MS), causes further problems, particularly when RPLC is coupled online to ICP–MS. These problems are detailed in Section II.C.3. The use of ion-pairing reagents allows RPLC to analyze ionic species. Additional advantages of this method are self-evident; for example, flexibility and variability are drastically increased. Also, very efficient separation is possible for a wide range of analytes, and the separation conditions may be tailored for the specific separation task.

Problems and limitations of ion-pairing RPLC are also increased in element speciation and hyphenation to, for example, ICP–MS. Again, organic solvents and acids might be used that could easily change element species. The structure of proteins may be destroyed, and complex-bound elements can be released to be recomplexed by other ligands; thus, species transfer reactions are likely. Covalent element-ligand bondings are best suited for analysis by this method. The use of ion-pairing reagents intensifies these problems even more, and analyte stability during separation becomes more difficult to maintain with IPC than with RPLC alone.

2. Capillary Electrophoresis

One of the most powerful separation techniques, capillary electrophoresis provides a very efficient separation of species and is often superior to LC separation techniques. CE is able to separate positive, neutral, and negative ions in a single run with high separation efficiencies. A single CE instrument can even offer several different modes of separation: capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), isoelectric focusing (IEF), isotachophoresis (ITP), and capillary electrochromatography (CEC). All of these modes rely on the

application of high voltage (Kuhn & Hofstetter, 1993); however, the separation principles are quite different, and they each provide a variety of characterization and identification mechanisms for elemental species. The latter point is of great importance, as species identification is rarely achieved by a single method; rather, it requires multidimensional strategies. Furthermore, species integrity is thought to be affected less than when HPLC is used. Limitations are seen in the very small sample volume used, thus giving rise to concerns with regard to the representativeness and homogeneity of the sample and instrumental detection limits (Michalke, 1999b).

3. Interfacing LC to ICP–MS

Combining separation methods with element-selective detection methods leads to hyphenated systems. These online systems give easier and faster results; thus the risk for contamination or loss is reduced. On the other hand, collected fractions allow for several quality control checks and application of the orthogonal identification concepts (Michalke & Schramel, 1997). Interfacing LC with ICP–MS is achieved by a nebulizer. Several pneumatic nebulizers are available, such as the Meinhard (MN), cross-flow (CFN), microconcentric (MCN), ultrasonic (USN), direct injection (DIN), and hydraulic high pressure (HHPN) nebulizers. Nebulization efficiency ranges from around 1 to 5% for the MN and CFN to quite high values for the DIN and USN (Dunemann & Begerow, 1995). The MCN and DIN are used for low flow rates between about 30 and $150\,\mu L\,min^{-1}$ and are believed to achieve nebulization efficiency up to 100%. These are suitable systems for interfacing with microbore LC.

The major problems with online hyphenation include:

1. The salt load of eluents can cause problems, such as crusting and changes in ionization.
2. The use of high amounts of organic solvents in RPLC cools the plasma and increases the reflected radiofrequency (RF) power. This results in plasma extinction already at relatively low organic solvent concentrations. Further, the ionization characteristics of the argon plasma are altered, which affects the sensitivity of the detector for element species. The extremely high carbon intake induces polyatomic interferences and carbon precipitation on the torch and cones. The conductivity of carbon can cause flashovers from the coil to the carbon-coated torch.
3. Sample transfer to ICP–MS; This may be influenced by dead volume of the interface, or affected by peak broadening and flow rate.

Developments to address the first issue listed above are typically based on novel column technology that provides high separation efficiency even at low (buffer) salt concentrations. The easiest way to overcome the problems caused by the second issue is post-column dilution of the eluent; however, this results in dilution of the separated analytes. The most common method to stand high organic modifier concentrations is to reduce the evaporation pressure of the modifier by cooling the transfer line and/or the spray chamber below 10°C. Methanol concentrations up to 60 to 80% in the eluent are tolerated at flow rates of about $1.5\,ml\,min^{-1}$. For improved burning of carbon from MeOH, the nebulizer gas is added with small amounts of oxygen. Desolvating systems are also used that can tolerate methanol concentrations up to 100% (Lustig et al., 1998); however, one should be aware that some species may be removed in the desolvator. Further aspects of nebulizer behavior are discussed by Montaser and Golightly (1992).

4. Interfacing CE to ICP–MS

Much effort has been devoted to interfacing CE with ICP–MS. It has been demonstrated that such hyphenated CE techniques can provide sub-$\mu g\,L^{-1}$ detection limits for the analysis of many types of environmental samples and are also capable of multiple-element monitoring of various metal functionalities. At present, an efficient interface is still unavailable. Furthermore, the tiny amounts of sample emerging from the capillary often give rise to detection limits that are usually higher than those of conventional chromatographic methods. An exhaustive discussion on CE–ICP–MS is given by Olesik (2000), who covers theory, application, and instrumentation of the procedure. One requirement for the interface is being able to close the electrical circuit from CE at the end of the capillary. The flow rate of CE generally does not match the flow rate needed for an efficient nebulization, but one possible way to circumvent this problem is to close the electrical circuit of CE during nebulization by applying a coaxial electrolyte flow around the CE capillary. The grounded outlet electrode is, in all cases, in contact with this electrolyte flow. The sheath flow has also been used to adapt the flow rate to a suitable nebulization efficiency. Optimization of nebulization efficiency was achieved by adjusting the flow rate when using MCN- or DIN-based systems or by exactly positioning the CE

capillary to the point of nebulization (*e.g.*, by employing a micrometer screw).

5. Interfacing CE to ESI–MS

Electrospray ionization (ESI) interfaces for CE are commercially available. The closing of the electrical circuit from CE during ion evaporation is provided by an electrolyte sheath flow. Effective ion production is made possible by the use of a suitable spray voltage easily controlled by the instrument software. For older instruments, a laboratory-made device for reproducibly optimized positioning of the CE capillary to the ESI tip is still necessary.

6. Gas Chromatography

The choice of an adequate separation technique is determined by the physicochemical properties of the analyte (volatility, charge, polarity), whereas that of the detection technique is determined by the analyte level in the sample. The combination of gas chromatography (GC) with ICP–MS has become an effective method for the speciation of organometallic compounds in complex environmental samples. GC separates volatile and gaseous element species employing primarily capillary columns with bonded phases. The big advantage is seen in the fact that species no longer have to be transferred into an aerosol or gaseous phase. Sample input into, for example, ICP–MS is about 100%, thus improving detection limits. Very often, however, species are not sufficiently volatile, and in such cases time-consuming and tedious sample preparation, extraction, and derivatization procedures are necessary, especially when carrying out a Grignard derivatization (Quevauviller *et al.*, 1996). This approach, on the other hand, removes the matrix components and makes separation easier.

Major problems are often encountered with derivatization, which is sometimes less selective than expected (Quevauviller *et al.*, 1996). The detector response is species-derivative specific, an important aspect to consider in quantification; however, some derivatives have nearly no detector response. The separation typically is carried out at elevated temperature; thus, only thermally stable species can be handled by GC. Another problem arises when coupling to element-selective detectors such as ICP–AES or ICP–MS. This coupling is not as straightforward as with LC and is affected by several limitations; for example, analytes have to be maintained in the gas phase during their transport from the GC to the ICP–MS to avoid condensation effects. The transfer line to the plasma should also be heated, either by a pre-heated sheath gas or by electrical

heating. The proximity of metal parts (heating wire) to the generator coil is problematic. Also, the effluent from the gas chromatograph (a few milliliters per minute) requires an additional carrier gas to achieve sufficient flow in the central channel of the plasma. For these reasons, most of the published papers devoted to the use of GC–ICP–MS address the construction and development of adequate interfaces. Typical applications of GC in speciation analysis are quantification of alkylated species of arsenic, mercury, lead, selenium, and tin.

7. Element-Selective Detection

Spectrometry-based techniques for the quantification of elements have long since taken root. Spectrochemical methods are frequently used because of their speed, detection power, sensitivity, and specificity. The impact that atomic spectrometric techniques in general have had on governmental institutions and international organizations has been carefully discussed by Minderhoud (1983), who made particular reference to the analysis of chemical wastes, sewage sludges, surface waters, and airborne particulates. The role of spectrochemistry in bioinorganic chemistry is still growing and will be even greater when chemical speciation can be fully accomplished.

Atomic absorption spectrometry (AAS) systems are comparatively inexpensive element-selective detectors that recently also matured from mono-elemental to multiple-element systems. There are flame (FAAS), cold vapor (CVAAS), hydride generation (HGAAS), and electrothermal atomization (ETA-AAS) AAS systems. The detection power of FAAS is often insufficient for normal environmental or physiological concentrations (Dunemann & Begerow, 1995). The sample intake is high (4–5 mL min^{-1}), which complicates online hyphenations with HPLC (optimized flow rates at about 0.5–2 mL min^{-1}); therefore, it becomes necessary to have an auxiliary flow, which results in analyte dilution. CVAAS and HGAAS use selective derivatization for matrix separation and detectability of relevant species. The detector response is strongly species dependent and often easily interfered. As an example, As species should be mentioned: if As(III) is assumed to have the highest response (100%), then As(V) is only about 85%, whereas arsenobetaine shows absolutely no response. ETA-AAS needs samples of only a few microliters and provides really low detection limits of between 0.1 and 5 µg L^{-1}. Matrix interferences are widely eliminated via Zeeman correction and matrix modifiers. However, quantification errors are still possible, as the final atomization temperature is only up to

2900°C. An optimized temperature program is part of the determination, which is the reason for a discontinuous measurement; therefore, ETA–AAS is unsuitable for online hyphenation to HPLC, as the chromatographic data points are gained only at intervals of a few minutes. This is too slow for peak description in a chromatogram. Moreover, during the temperature increase for sample drying, volatile species may be lost before being atomized.

Commonly used alternatives to AAS-centered approaches include ICP–AES and ICP–MS detectors. The special diagnostic advantage of plasma-based techniques is their rapid screening ability, which has often confirmed suspected heavy metal poisoning (Ure et al., 1993), mineral deficiencies, or storage diseases. Equally as often, however, these techniques have identified completely unsuspected etiologic factors. Barnes (1991) surveyed the potential of ICP–AES combined with flow injection analysis, direct sample introduction and vaporization systems, electrothermal vaporization, hydride, metal vapor and gas generation, and chromatographic techniques. The big advantage of ICP–AES is offered by its multiple-element capability and sensitivity. Online hyphenation is easily set up. The ionization source is an inductively coupled plasma, and the temperature is around 5000 to 9000 K. The plasma (mostly argon) is formed within a quartz torch made up of three concentric quartz tubes, with the gas flowing at different rates through each. The outer flow is the highest and is known as the "plasma," "coolant," or "support" gas flow. It is tangentially introduced into the plasma torch to provide a helical flow that sustains the plasma itself. The central gas flow, known as the "auxiliary" gas flow, keeps the plasma away from the edge of the quartz torch. The inner gas flow, commonly called the "nebulizer" gas flow, transports the nebulized samples to the plasma. An RF field provides the energy to sustain the argon plasma, and the plasma transfers energy to the analyte(s) for excitation and ionization. Excellent discussions of plasma theory, mechanism, and applications are given by Montaser and Golightly (1992). Chemical interferences such as molecular emissions cause no major problems, but background correction should be applied in any case. Sample introduction is performed via a nebulizer and spray chamber. Nebulizer types, related problems, and some solutions have already been discussed earlier. Spectrometers might be of the sequential or simultaneous type. For on-time, multiple-element monitoring, simultaneous ICP–AES systems are used, as the sequential type results in a loss of chromatographic datapoint resolution (similar to ETA–AAS). Recently, ICP–AES systems equipped with

a charge-coupled device (CCD) helped to overcome this problem.

Inductively coupled plasma–mass spectrometry is now the technique of choice for a wide range of samples with element concentrations in the $\mu g\, g^{-1}$ to sub-$ng\, g^{-1}$ range. It has become a highly versatile technique with low detection limits and high sensitivity. Also, thanks to its element specificity, it is a technique of choice for chromatographic detection, including GC, LC, SFC, and CE. The striking advantages of ICP–MS techniques are their detection selectivity, multiple-element capability, and high sensitivity. Isotopic and elemental information of species is obtained, and species not totally resolved but pertaining to different elements are distinguished by the selective detector.

Sample introduction is performed through an interface connecting the LC system to the ICP–MS. The availability and pros and cons of such interfaces (various types of nebulizers) have been discussed previously. In hyphenated systems, detection limits for element concentrations in elemental species were reported to be in the range of 10 to $100\, ng\, L^{-1}$ (Olesik, 2000; Michalke et al., 2001). ICP–MS can be of the quadrupole type (Q-ICP–MS) or the highly resolving sector field type (SF-ICP–MS). The latter is reported to improve detection ability by a factor of 10 to 100, or even up to 1000 when equipped with a guard electrode (Prange & Schaumlöffel, 1999). The quadrupole mass filter provides a resolution of only 1 amu; therefore, polyatomic interferences are likely to occur, especially in the mass range of 40 to 80 amu. Well-known interferences are those of the $^{40}Ar^{35}Cl^+$ double ion on monoisotopic ^{75}As or of $^{40}Ar^{12}C^+$ on ^{52}Cr (Tittes et al., 1994). They are produced in the argon plasma when chlorine and carbon are introduced into the plasma (sample and buffer components, respectively). The highly resolving SF-ICP–MS can distinguish between the interference and the element isotope, as the mass resolution is 7500 to 10,000 amu (as compared with 300 amu for Q-ICP–MS). An example for such separation is given in Figure 2.

When using SF-ICP–MS at a high mass resolution, the detection power is reduced, and it falls into the same range of Q-ICP–MS. With element-selective detectors such as ICP–MS, one has to realize that only the element in the species is detected, not the entire molecule, which is advantageous because the separation of molecules bound to different elements does not need to be complete. They may be screened by the detector responding to different isotopes; however, it must be kept in mind that the molecule itself is not seen, and the species identification is only possible by comparing
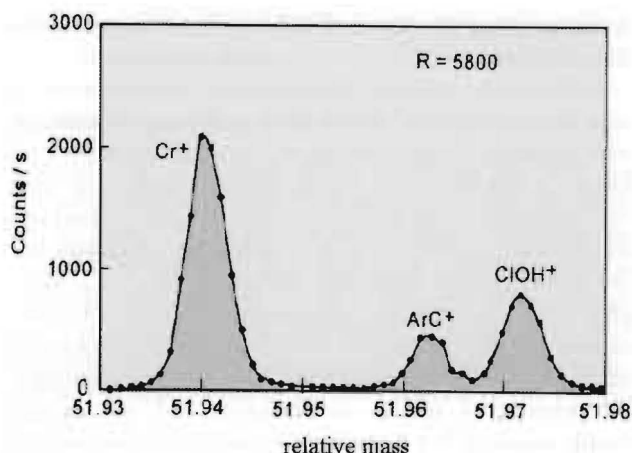
FIGURE 2 ICP–MS detection may be affected by, for example, polyatomic interferences. Here, the resolution of $^{40}Ar^{12}C^+$ interference and ^{52}Cr by sf-ICP–MS is shown. (From Tittes, W. et al., Reduction of Some Spectral Interferences in ICP–MS, Finnigan MAT Elemental Mass Spectrometry Technical and Application Note 3, Finnigan MAT GmbH, Bremen, Germany, 1994. With permission.)

retention times. In natural samples, this is not always achieved beyond doubt; therefore, very often speciation analysis with only, for example, LC–ICP–MS is not enough for unequivocal speciation results. Multidimensional analytical concepts are strongly indicated in such cases (Michalke, 1999a).

For the identification of polyatomic interferences, the monitoring of several isotopes of a given element can be helpful. Only when the natural isotope ratio is measured can interferences be ruled out. Unsatisfactory sensitivity is still a problem for very low concentration samples; hence, monitoring the most abundant isotopes of an element is recommended, except when these isotopes are interfered (e.g. poly-atomic interferences). On the other hand, ICP–MS is a sequential detector that monitors the programmed isotopes for several milliseconds. If too many isotopes are programmed for subsequent determinations, the detector operates too slowly to allow for highly resolved and fast-appearing peaks on one specific mass. This causes a loss in chromatographic resolution of the hyphenated system. The recently available time-of-flight (TOF) ICP–MS can overcome this drawback, as the mass filter does not jump in a time-consuming manner from one mass to another. Instead, the different isotopes are distinguished as a function of their individual (m/z-dependent) time to reach the detector.

8. Species-Selective Detectors: Electrospray Ionization Mass Spectrometry Detection

Electrospray ionization is a process that may preserve the whole species intact under optimal circumstances. ESI is suitable for extremely low flow rates. It is based on the so-called ion evaporation principle, where charged droplets of the analytes are transferred into the gas phase. A volatile buffer consisting of considerable amounts of, for example, methanol supports the ion evaporation process. In fact, the high volatilization capability of CE electrolytes is mandatory. The success of this detection method is based on the ability to produce multicharged ions from high molecular element species, such as metalloproteins, thus making the analysis of these compounds feasible up to molecular weights of 150,000 to 200,000 amu. The possibility of coupling this detector to LC or CE systems makes it extremely valuable. The soft ionization of element species finally allows preservation of the entire molecule (element species) when it is transferred into the gas phase and subsequent analysis by mass spectrometry (Cole, 1997). Structural changes normally do not appear as long as covalent bonds are present. In special cases, stable element–organic molecules can be analyzed.

When applying collision-induced dissociation (CID) together with an MS/MS system, further structural information can be gained as the parent ions are fragmented into molecule-specific daughter ions, which are then selected by the second quadrupole. No other detection technique is able to provide such detailed information about molecular weight and even structure of the analyzed compounds. On the other hand, significant problems have been described for ESI. One problem arises due to the ion–solvent clusters. During the transfer of gas-phase ions into the high-vacuum (10^{-9} bar) zone, condensation of solvent molecules (e.g., methanol, water) from the gas-phase ions is likely to happen. This is what is referred to as an ion–solvent cluster and is caused by the cooling-down phenomena that occur when a gas expands into a vacuum (i.e., free jet expansion, adiabatic expansion). Ion–solvent cluster production results in the splitting up of one species into multiple signals, worsening detection limits, and increasing spectral complexity. Electrolytic processes at the metallic ESI tip needle that can generate new species or transformations of species (e.g., by metal exchange) are also observed. When analyzing free metal ions such as Cu(II), multiple ion–solvent cluster signals are again detected. Most important, however, is the fact that native counter-ions of the metal ion are replaced

by H_2O and/or methanol, independent of the counter-ion initially present (*e.g.*, $[Cu(MeOH)]^+$) instead of $Cu^{2+}2Cl^-$). This implies a total loss of the original species information.

III. QUALITY CONTROL IN SPECIATION

Causes of disagreement may be traced back to poor methodology, improper instrument calibration, faulty experimental techniques, impure reagents, or a combination thereof. Because of the lack of reliable data on trace elements in biological fluids, the reported diagnostic significance of some elements is controversial. The same holds for inaccurate measurements of pollutants in environmental matrices such as sediments, water, and particulate matter. Extremely sensitive instrumentation is readily available in laboratories not equipped to control contamination, and many users of a particular technique do not fully understand the limitations of a methodology. All of these factors introduce a great deal of questionable data. Much work on trace elements in human body fluids and tissues has suffered from methodology deficiencies, but accuracy is needed to reach rational conclusions upon which basic healthcare decisions are built. A rigorous program of quality control/quality assurance is needed to confirm the reliability of results for trace elements in biological materials.

A. General Aspects of Quality Control

Quality control plays a crucial role in element speciation. General hypotheses and analytical models depend on the reliability of data (Quevauviller *et al.*, 1996). The key to successful speciation is the preservation of species information during the whole analytical procedure from sampling to final analysis. Actually, this is rarely guaranteed, and the range of errors is extremely high; thus, quality control is needed in the planning stages of an experiment. Pertinent literature must be sought and general criteria must be adapted to the actual problem relevant to the elements and matrices of interest.

All trace and ultratrace analyses require clean workplaces and the continuous use of appropriate control materials. These prerequisites are absolutely necessary for reliable results, and determination of the blank level will be that much more accurate. If these rules are not strictly followed, the money and time spent are completely wasted. For work with trace elements (and their chemical species) at very low concentration in biological materials, clean-room techniques may become mandatory. Other prerequisites are listed below:

1. *Calibration*—A necessary prerequisite of reliable analytical work is correct calibration using calibrants of each investigated species with known stoichiometry. Preparation of two parallel calibrant solutions should be done according to weight and not volume. High purity of chemicals is compulsory. New lots of calibrants must be verified. Calibration graphs of the single species must be generated.

2. *Quantitative speciation*—Species quantification is the basis for obtaining mass balances and gaining information about losses and contaminations. Quantification must be done using the relevant calibration curve (or standard for standard addition). If unknown compounds are monitored, quantification is not possible. Species can only be estimated by relating peak area calibration graphs to those of closely eluting known species.

3. *Certified reference materials*—One of the favored approaches for quality control is the use of reference materials (RMs) or certified reference materials (CRMs), although in environmental analysis there is a dismal lack of RMs to match real-world situations. The somewhat restricted market is certainly partially responsible for this. The introduction of more stringent regulations will enhance the demand for RMs. Operational complexity associated with the production of environmental RMs and the amounts required (5000–1000 kg) are also paramount factors.

Blending is an alternative procedure to prepare RMs of intermediate concentrations. Strategies to ensure accuracy include: (1) building accuracy into RMs under the guidance of a few centrally operating agencies, (2) transfer of accuracy from RMs to the measurement system, and (3) safeguarding the accuracy levels by continuous measurement quality control. Political and economical decisions concerning the environment are based on the correctness of analytical data. The last 30 years are testament to a growing awareness of the mistakes and pitfalls that accurate RMs should help eliminate. Suitable RMs and CRMs should be included at the earliest possible stage in any speciation analysis process. Confirmation of the certified value can prove the reliability of the results for unknown samples (Quevauviller *et al.*, 1996); however, doing so never gives full evidence

TABLE IV.   General Criteria for Speciation Analysis

Sampling	Extent to which the samples are representative should be verified (Quevauviller et al., 1996).
	Sampling time should be kept short.
	Contamination phenomena should be minimized.
	Volume-to-surface ratio should be high to reduce wall effects.
	Use of stainless steel tools is not advisable when sampling biological materials (Dunemann & Begerow, 1995).
Sample preparation	Short storage at °C or freeze-drying (possible loss of volatile species) is recommended.
	Extractions typically result in operationally defined speciation.
	Mass balances and recovery rates (spiking of species) should be determined; species spiked can exhibit different extraction behavior.
	Species spikes endanger native equilibria in the sample and could lead to a changed species pattern; comparison of different extractions is the best way to get reliable information (Quevauviller et al., 1996).
Derivatization	Derivatization should be avoided; it increases detection power but can affect the species.
	Selectivity is much lower than expected unless other options are available.
	Different efficiencies of derivatization and different detector responses of the derivatives are observed to some extent (Quevauviller et al., 1996).
Separation	Stationary phases may cause contamination and retain undesirable reactive groups.
	Contamination or stability problems of species occur; these undesirable effects should be monitored by mass balances or checked by reinjection experiments (Michalke & Schramel, 1997); potential species transfers should be investigated and possibly excluded.
	Identification problems arise when the identity of a species is only attributed by retention times in a low resolving separation system.
	Multidimensional analysis using various independent methods is the best alternative (Michalke, 1999a; Szpunar, 2000b).
Detection	Nonspecific detection should be avoided.
	Calibration should be done properly.
	Suitability of the detector for the problem at hand should be checked.
	The discontinuous measuring nature of ETA–AAS results in too low chromatographic resolution; quadrupole ICP–MS exhibits maximum mass interference in the m/z range of 40–80.
	Several isotopes of the same element should be monitored to check polyatomic interferences.
Multidimensional analytical concepts	Combining various separation techniques and detection systems is recommended.
	Two schemes are generally employed:
	1. Separation methods based on different separation principles are combined. After the first separation, fraction collection provides aliquots to be used for element determination and as samples for the second separation, which is again followed by online ICP–MS monitoring. This procedure provides an orthogonal characterization of molecules.
	2. Separation is monitored in parallel by ICP–MS and ESI–MS. In this case, ICP–MS provides the element information and ESI–MS (or MS/MS) gives molecular or structural information; hence, obtaining maximum species information and sometimes identification becomes possible. These schemes can also be combined in different ways (Michalke, 1999a; Szpunar, 2000b).

of the correctness of results, as sample matrices are rarely identical to the CRM matrix. The concentration range of the species or the species pattern may be different, which results in variation in their behavior during the analysis (Quevauviller et al., 1996). Many CRMs are available—for example, from the National Institute of Standards and Technology (NIST) in the United States, Institute for Reference Materials and

Measurements (IRMM) in the EU, and National Research Council (NRC) in Canada. In practice, however, many other precautions are necessary, as summarized in Table IV.

Preservation of samples for future controls and investigations into their content with regard to unsuspected chemical species are well-recognized needs. Sample banking is a new concept that involves the preservation
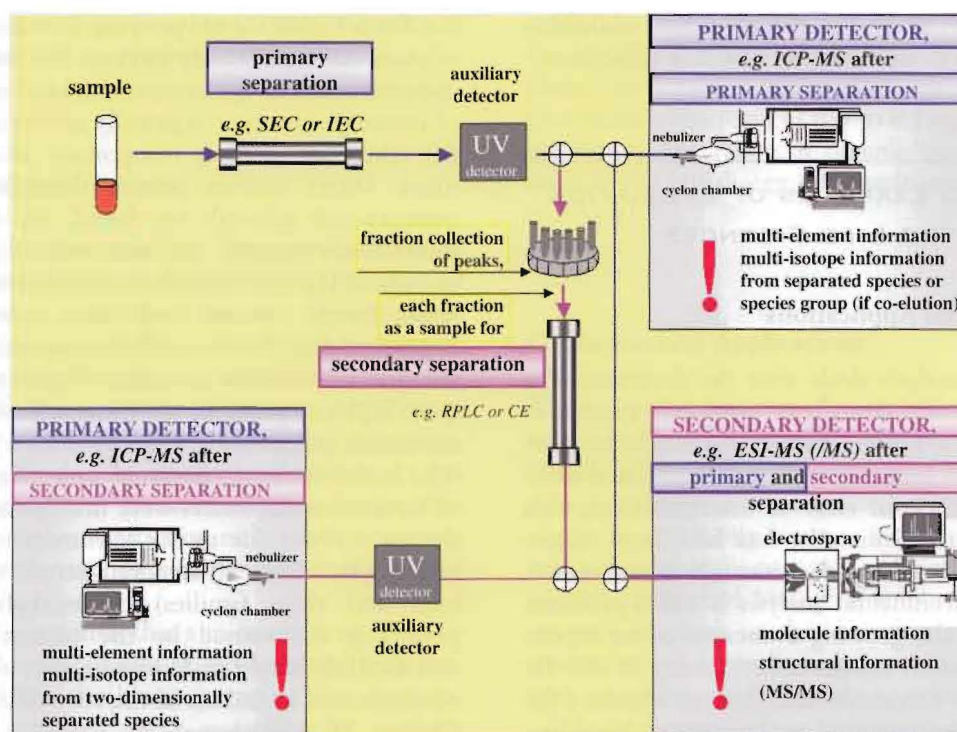
FIGURE 3 The principles of an orthogonal (analytical) speciation concept are shown. General advantages include the ability to obtain multi-element/multi-isotope information using element-selective detection after the first species separation combined with molecular (and probably structural) information provided by a second molecule-specific detector. In case of coelution after the primary separation step, further purification is achieved by a secondary separation step, again providing multi-element/multi-isotope and molecule-selective information. Clear species identification is usually possible.

of important samples under unequivocal conditions that ensure their integrity over extended periods of time, but sample banking is expensive.

B. Orthogonal Analytical Concepts

Analytical strategies that employ combinations of various separation and/or detection methods are referred to as *orthogonal analytical concepts*. They are an indispensable means for quality control in speciation and offer the best opportunity for obtaining accurate speciation results and even identification of unknown species. In analytical systems with (only) one separation and one detection system, the risk of coelution, impossibility of species identification, or misidentification is high (McSheehy et al., 2002). This problem can be solved by employing different systems in various ways.

In short, these multidimensional analytical concepts rely upon combinations of various separation technologies and detection systems. Two schemes are primarily employed:

1. Two separation methods based on different separation principles are combined in a series. After the primary separation, fraction collection provides aliquots to be used for element determination and as samples for the secondary separation, which is again followed by an (online) ICP–MS monitoring. This results in an orthogonal characterization of molecules.
2. The separation is monitored in parallel by ICP–MS and ESI–MS. Here, ICP–MS provides the element information while ESI–MS (or ESI–MS/MS) delivers the molecular or structural information; hence, maximized species information and sometimes identification are possible.

Often, it is necessary to combine the two schemes in different ways (Michalke et al., 1997; Chassaigne et al., 1998; Michalke, 1999a; Szpunar, 2000a). Figure 3 provides an orthogonal flow chart, and a current example has been published by McSheehy et al. (2002). Similarly, Nischwitz et al. (2003) checked species preserving extraction techniques using an orthogonal scheme, first using SEC with ICP–MS, fraction collection with sub-

sequent RPLC–ICP–MS, and, finally, after collecting cleaned fractions from the latter, ESI–MS detection.

IV. SELECTED EXAMPLES OF SPECIATION STUDIES ON THE LIFE SCIENCES

A. Environmental Applications

Environmental analysis deals with the detection of a variety of substances naturally or artificially present in our total environment and known or thought to exert adverse effects on human health. Environmental measurements are a special class of determinations with common problems. Ordinarily, data have large uncertainties, and the resulting decisions are controversial. Accuracy in environmental analysis is still a problem, notwithstanding the growing awareness of its importance. Trace element measurement quality is still far from satisfactory. Environmental data are obtained for the purpose of information and/or action. Since the industrial revolution, mankind has continued to pollute the Earth's biosphere with toxic substances such as heavy metals and halogenated organic compounds. Many of these substances are not amenable to processes that would lead to their removal; as a result, many industrial chemicals build up in the environment. During the past 100 years, the environment has been severely polluted by arsenic, cadmium, mercury, lead, and thallium. The increasing interest of governmental agencies, particularly the Environmental Protection Agency (EPA), in ICP–AES as a novel and useful alternative to established procedures for monitoring elements in environmental media was stressed 20 years ago in a report on the potential future of this technique (Barnes, 1983).

Large amounts of impurities (both natural and industrial) can be found in the atmosphere. Some of them form flying ashes and other gaseous emissions with numerous elements, the toxicity of which depends on their chemical species. Limit values in regulations must be checked regularly, and levels of pollutants must be known in order to identify systematic changes and pollution sources.

The well-documented risks posed by concentrations of mercury in the environment are caused by human activity. Although the annual world consumption of mercury by industry is estimated to be around 10,000 tonnes, the total worldwide release of mercury as a result of human activities has been estimated to be 20,000 to 35,000 tonnes per year. It is unlikely that such releases can significantly increase the average mercury concentrations in the oceans, although increased levels of mercury can occur regionally and locally because of the release of mercury compounds into the environment. Water courses passing through areas where mercury-rich minerals are found (the main mercury minerals are cinnabar and metacinnabar, the two polymorphs of HgS) are known to result in elevated mercury levels. In the aquatic food chain, mercury plays an important role. Because of bioaccumulation processes, the mercury content in marine organisms is normally quite high compared to the content in water, and concentration factors of 10^4 and higher have been reported. The health hazards associated with mercury pollution of environmental waters were first brought to light in the early 1960s due to the Minamata mass poisoning catastrophe of several hundred people (mainly fishermen and their families) by methylmercury. The poisoning was caused by the consumption of fish and shellfish caught in Minamata Bay, which had been contaminated by industrial mercury discharge (see also Chapter 23, this volume).

Three categories of methodologies for environmental analysis can be defined as: (1) definitive techniques of high precision and accuracy—and high cost; (2) more routine techniques to be used for continuous monitoring of exposure to pollutants; and (3) field methods for preliminary semiquantitative assessment under emergency circumstances requiring urgent countermeasures.

Marine- and freshwater have been significantly contaminated by organotin compounds, such as mono-, di-, and trisubstituted butyltin and phenyltin, because of their use as agrochemicals, biocides, and domestic products. The performance of four specific devices—namely, flame photometric detector, pulsed flame photometric detector, microwave-induced plasma atomic emission spectrometer, and inductively coupled plasma mass spectrometer—was compared in terms of ability to quantify the species of interest after separation by means of solid-phase microextraction and GC (Aguerre et al., 2002). The different approaches showed that the determination of ultratrace tin species is possible in natural waters even for routine purposes. Detection limits as good as $0.5\,\mathrm{ng\,L^{-1}}$ tin could be reached.

A detailed description of the complexation chemistry of copper in natural waters, with particular reference to water quality criteria and to possible effects on biota, was reported by Allen and Hansen (1986). It was concluded that the bioavailability and toxicity of Cu depend primarily on pH and alkalinity. When these two parameters are constant in a given system, then bioavailabil-

ity and toxicity are proportional to the concentration of free copper ions and inorganic Cu complexes.

B. Nutritional Applications

It is a generally valid principle that each nutrient at excessive concentrations can be toxic; conversely, any of the trace elements now known for their toxicity might be shown in the future to have an essential function at low concentrations. Among the trace elements of nutritional interest are arsenic, chromium, manganese, molybdenum, nickel, selenium, silica, tin, vanadium, and perhaps cadmium. These elements present serious problems of analysis in the concentration ranges that are of interest to the nutritionist, either as toxic species even at low concentrations (*e.g.*, As (III)) or as essential element species.

The initial recognition that a micronutrient is essential for animal species raises questions about its nutritional and public health importance for humans. Nutritionists approach these questions by working through some specific tasks, such as identification of the metabolic parameters in humans that might be affected by the micronutrient, detection of disease states that can be prevented or cured by supplementation, determination of the human requirement for the micronutrient, assessment of the risk for dietary imbalances in population groups, and, finally, development of methods for assessing the nutritional status in individuals. All of these tasks, except for the last one, have been more or less accomplished.

Zinc, for example, plays a key role in erythrocyte carbonic anhydrase, an enzyme catalytically involved in the transport of CO_2 in blood. From a nutritional viewpoint, it is interesting that zinc deficiency is typically seen in population groups living in poverty. Consumption of vegetarian phytate-rich food is common and provides zinc–phytate complexes (species) of reduced bioavailability (Brätter *et al.*, 1992). An analogous situation is known from formulas for newborns as compared to human milk. Formulas based on cows' milk contain higher amounts of casein, calcium, and phosphorus. These compounds together associate to casein–phosphorus–zinc micelles of low availability for zinc. Contrary to this, human milk shows zinc–citrate complexes that are easily accessed by the newborn's gut.

The ability of surfactants to differentiate between methylmercury and inorganic Hg(I) in fish-egg oil was ascertained by using an online, time-based injection system in conjunction with CVAAS (Burguera *et al.*,

1999). An advantageous flow could be obtained from the highly viscous sample by injecting it into a three-phase surfactant (Tween 20®)–oil–water emulsion. Quantities of mercury as low as $0.1\,\mu g\,L^{-1}$ could still be measured. The range of organic mercury concentrations in the catfish-egg oil sample was found to be 2.0 to $3.3\,\mu g\,L^{-1}$.

C. Biomedical Applications

The overall importance of analytical atomic spectrometry in biology and medicine was reviewed by Dawson (1986). While this author recognized the fundamental role played by ICP–AES and other spectroscopic techniques in generating accurate and reliable data, he also emphasized that the next step in the analysis of elements of well-established biological importance would be a growing emphasis on the identification and quantification of their biologically active species through coupling with adequate online separation procedures.

The importance of trace elements and the identification and quantification of their chemical forms cannot be overestimated. Some of the elements essential to humans (cobalt, chromium, copper, iron, iodine, manganese, molybdenum, nickel, selenium, silica, and zinc; see Brätter *et al.*, 1992) have a vitamin character. Others are present in pharmaceuticals as active agents (aluminum, arsenic, gold, bismuth, copper, iron, mercury, lithium, platinum, and zinc). Uncontrolled use of pharmaceuticals can lead to undesired effects and intoxications, thus requiring immediate control therapy.

The human requirement for chromium is lower than that for any of the essential trace elements, except for cobalt as a part of vitamin B_{12}. Chromium is not only an essential ultratrace element but also a potent carcinogen. Many other essential trace elements are also toxic at excessive exposures, but chromium is unique in that its essentiality is limited to one valence state and toxicity to another, and transformation from the essential to the toxic valence state does not occur in the living organism. Those and other properties of chromium that influence its interaction with biological systems reside in its chemical behavior. Chromium in the trivalent state is an essential element for animal species and humans; in the form of certain hexavalent compounds it is a potent carcinogen. Hexavalent compounds are manmade and do not occur naturally in living organisms. They penetrate biological membranes and are reduced by organic matter, which leads to oxidative damage of cell structures. While there is little evidence

for a role of Cr(III) in enzyme systems, its interactions with nucleic acids and with the functions of the thyroid gland and of insulin have been demonstrated. The lack of a simple diagnostic procedure, which chromium shares with many other essential trace elements, is the main impediment to the wide application of chromium in medicine and nutrition. Without diagnosis of chromium status, the response of an individual to supplementation is unpredictable.

Among the essential trace elements, selenium is receiving increasing attention as a natural cancer-preventing agent. The anticarcinogenic effects of selenium have been demonstrated in numerous animal tumor model systems as well as under conditions simulating human dietary conditions (Patching & Gardiner, 1999). Because the positive effects of selenium on Kashin-Beck disease are known, its protective mechanisms on several heart diseases, predominantly cardiovascular damage or congestive cardiomyopathy, have been widely investigated (see also Chapter 15, this volume). Detoxification effects of selenium have been proven and described for various metals such as arsenic, thallium, silver, cadmium, and mercury. Selenium deficiency is most critical for the brain and growth of infants. Furthermore, the thyroid metabolism may be impaired, because many deiodinases are Se proteins. These positive effects of selenium species led to several studies on selenium speciation in supplements, food (Quijano et al., 2000), and body fluids such as human milk (Michalke & Schramel, 1997) and serum. The selenium speciation in serum is expected to mirror the available concentrations of relevant protective selenium species. As an example, Figure 4 shows various chromatograms monitoring ^{82}Se after SAX separation from children's sera. Although at present not fully explained, these chromatograms from the sera of children with absorption abnormalities in the gastrointestinal tract show different patterns of selenium species as well as different total selenium levels. Gaining a full understanding of possible interrelations between alterations in the gut and the selenium species pattern will be a demanding task in the future.

Iodine, long known to act beneficially in human health as an essential micronutrient, is utilized by the thyroid gland for the biosynthesis of the thyroid hormones thyroxin (T_4) and triiodothyronine (T_3) (Keller, 1991) (see also Chapter 5, this volume). These hormones strongly influence an extended range of biochemical reactions. Immune defense and antibody production are dependent on reliable thyroid function and the availability of T_4 and T_3 hormones. The speciation of various iodine species in serum or urine provides information about malfunction of the thyroid gland and can also explain other T_4/T_3-influenced metabolic abnormalities. The superiority of hyphenated techniques with ICP–MS (iodine-) detection over monoclonal antibody systems has recently been demonstrated, as the monoclonal antibody systems were generally unable to distinguish between iodinated (active) and non-iodinated hormones (Michalke et al., 2000). Recently, iodine speciation was reported during investigations into the disruption of normal thyroid function by xenobiotic chemicals.

The increased rate at which zinc-containing metalloenzymes have been identified in the past is largely due to the development of highly precise, rapid, and convenient methods for determining this element. Definitive knowledge that zinc is indispensable to living matter has emerged only in the last few decades. In succession, the biological effects of zinc have been viewed as mostly harmful, then questionable, then essential; however, it is now well established that zinc is essential for the growth and development of all living forms. There is a lack of reliability in the techniques for aluminum determination that can affect a large number of samples encountered in clinical practice. Better measures for the quality control of such determinations have progressively led to improved detection limits for aluminum in biological material, thus paving the way to more reliable figures for the concentrations of their species of clinical relevance. Human serum was incubated in vitro with the radiotracers ^{51}Cr(III), ^{191}Pt(I), or carrier-free ^{48}V(V) (Lustig et al., 1999), and the protein-bound metals were measured by flatbed electrophoresis followed by autoradiography with laser densitometry, followed by subsequent detection of the proteins through silver staining. At this stage of its development, however, this proposed technique, although highly promising, does not possess adequate detection power.

V. SUMMARY

The future of speciation analysis depends to a large extent on three major factors: (1) development of instrumentation actually designed for this purpose (and not simply assembled from apparatuses originally conceived for another purpose), (2) production of a substantial number of CRMs especially prepared for chemical species (and not only the total content of a given element), and (3) ability to transfer the relevant know-how from the expert laboratory to the routine
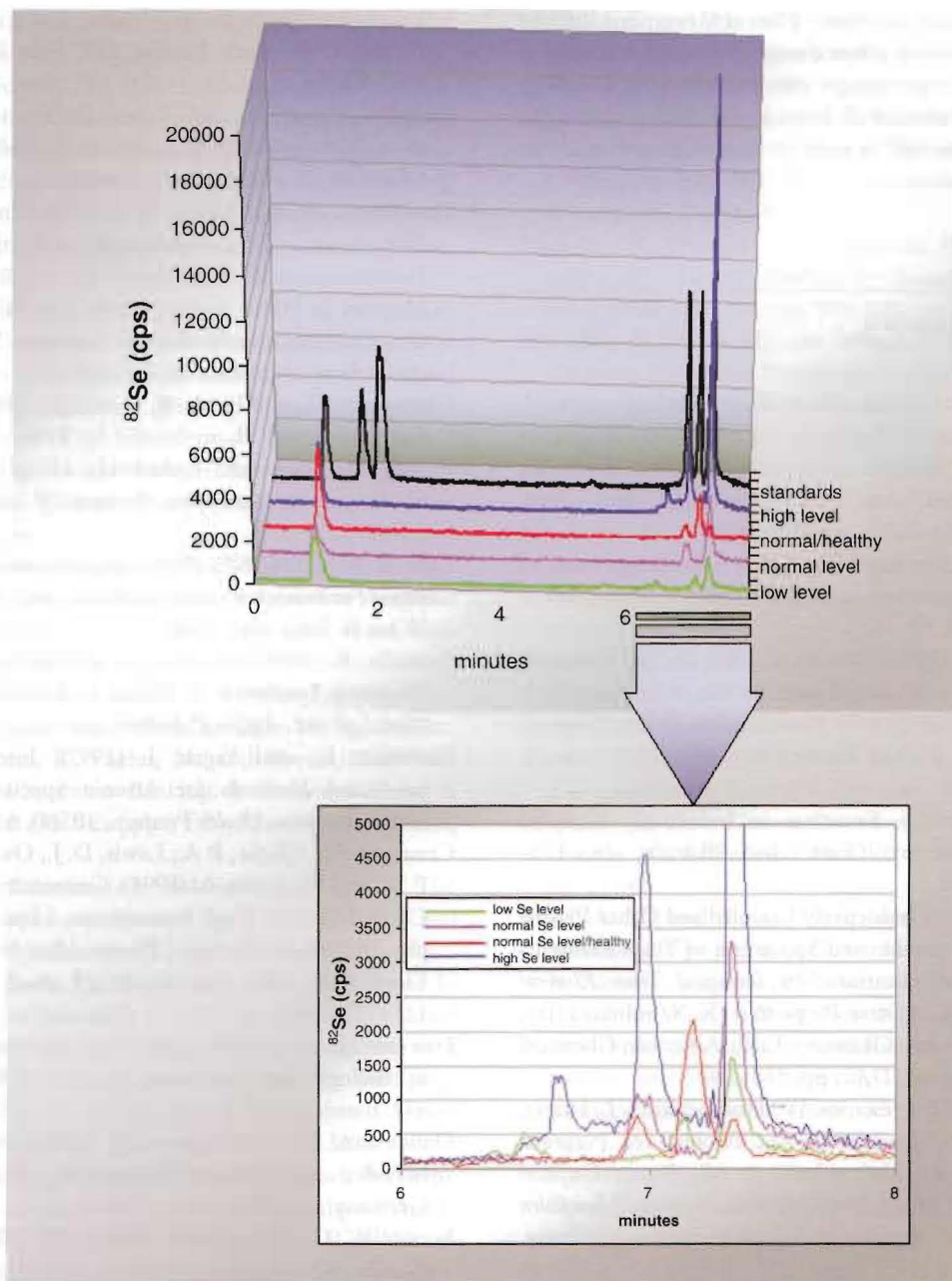
FIGURE 4 Chromatograms of children's sera ^{82}Se after SAX separation are shown (with the region between 6 and 8 minutes retention time additionally magnified). These sera from children having different absorption abnormalities in the gastrointestinal tract show different patterns of Se species as well as different total Se levels. For comparison, a 20-µg Se/L standard chromatogram is also plotted.

laboratory. Currently, the most suitable speciation analysis combines systems primarily based on the hyphenation of separation technologies online with element- or molecule-specific detectors. The variety of separation principles available today allows the separation of most species present in liquid samples. The selective detection ability of ICP–MS provides only signals of interest (not all compounds can be resolved) with very high detection sensitivity; however, there are still several pitfalls, so strict quality control and quality assurance schemes must be implemented. In speciation analysis, one of the most promising approaches is based

on the orthogonal scheme. The achievement of the above three goals will allow decision makers to develop regulations incorporating more effective, current knowledge on chemical elements and their role in the life sciences to the full benefit of human health and the environment.

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